# THE EFFECT OF NEONICOTINOID PESTICIDES ON NON-TARGET ORGANISMS

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**Thesis submitted for the degree of Doctor of Philosophy**

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**May 2018**

*“Live as though you’ll die tomorrow, but farm as though you’ll live forever”*

- *John Marsden*

**ABSTRACT**

There is widespread concern over the use of neonicotinoid pesticides in agro-ecosystems, and their effects on the wider environment. This is due in part to their high solubility in water which can lead to widespread contamination of non-target areas including standing surface water, soil, and non-target vegetation. The contribution of neonicotinoid exposure to the ongoing wild pollinator population declines has been the focus of considerable in-depth recent research, focused on the impacts on honeybees, bumblebees, and more recently on solitary bees. However, relatively little research has examined the impacts of exposure on other beneficial non-target organisms. This thesis investigates the impact of field-relevant concentrations of two neonicotinoids: thiamethoxam and clothianidin, on a range of non-model organisms. Model systems were developed for laboratory based experiments on three species: the **hoverfly** *Eristalis tenax;* the **butterfly** *Polyommatus icarus;* and the **earth worm** *Lumbricus terrestris*. A further semi-field experiment investigated the colonisation of contaminated microcosms by **aquatic invertebrates**. An additional review and analysis of UK time series data tested the relationship between agricultural change (including neonicotinoid usage) and changes in multi-species **farmland bird** populations.

Principally, the results corroborate previous research on non-target organisms and neonicotinoid exposure, showing a negative effect on mortality, food consumption and growth across a range of organisms. Clothianidin decreased the survival of *Lumbricus terrestris,* exposed via treated soil. Field-realistic exposure also had a significant but temporary effect on food consumption. Sublethal – and sometimes lethal – impacts of clothianidin were also found on the larvae of *Polyommatus icarus*. Both clothianidin and thiamethoxam showed significant negative effects on Diptera and Ostracoda, with clear differences between the effects of the two chemicals. In contrast, the larval stage of the hoverfly *Eristalis tenax* was unaffected by field realistic doses of thiamethoxam, with no observed effects on survival or development, nor showed any latent effects on adult activity budgets resulting from exposure to lower concentrations. The re-analysis of the relationship between agricultural change and bird population changes confirmed that evolving agricultural practices continue to affect farmland birds; a moderately significant negative relationship was found between bird population change and neonicotinoid exposure risk.

This thesis shows that neonicotinoids have a range of varying and unpredictable negative impacts on diverse invertebrate taxa; and demonstrates that it is possible to develop new model systems to test the effects of pesticides on often-overlooked taxa.

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DECLARATION

The thesis conforms to an 'article format' in which the middle chapters consist of discrete articles written in a style that is appropriate for publication in peer-reviewed journals in the field. The first and final chapters provide overview and discussion of the research undertaken.

**Parts of this work have been published as:**

Basley, K., Davenport B., Vogiatzis, K., and Goulson, D. (2018). Effects of chronic exposure to thiamethoxam on larvae of the hoverfly *Eristalis tenax* (Diptera, Syrphidae). PeerJ 6:e425 doi.org/10.7717/peerj.4258 (Chapter 2)

Basley, K., and Goulson, D. (2017). Effects of chronic exposure to clothianidin on the earthworm *Lumbricus terrestris*. PeerJ 5:e3177; DOI 10.7717/peerj.3177 (Chapter 3)

Basley, K., and Goulson, D. (2018), Neonicotinoids Thiamethoxam and Clothianidin Adversely Affect the Colonisation of Freshwater Microcosms. Environmental Science and Pollution Research. DOI 10.1007/s11356-017-1125-5 (Chapter 4)

Basley, K., and Goulson, D. (2018), Effects of field-relevant concentrations of clothianidin on larval development of the butterfly *Polyommatus icarus* (Lepidoptera, Lycaenidae). Environmental Science & Technology, 52, 7, 3990-3996. DOI 10.1021/acs.est.8b00609 (Chapter 5)

Chapter 6 is written in the style of an article appropriate for PeerJ

**I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.**

Signature:.....

# ACKNOWLEDGEMENTS

I would like to thank the numerous people that have made this work possible. Firstly, I would like to thank my supervisor Professor Dave Goulson for his patience, support and for advising me through the maze that is publication.

My wonderful lab group, who were there to provide a breath of fresh air and advice. They didn't judge me when I wandered around the department covered in dead worms and stinking of silage! So, thank you to Linda Birkin, Ellie Rotheray, Cristina Botías Talamentes, Tom Wood, Saija Piiroinien, Andreia Penado, Rob Fowler, Janine Griffiths-Lee, Science Mum, and Beth Nicholls – I feel so proud to know you all and acknowledge all the wonderful work you do. Many thanks also to Jerry Wilson from the RSPB for his support.

Thank you to my funders at the Natural Environment Research Council and the British Trust for Ornithology for providing bird population data and many thanks to Dr Peter Shaw who helped with some beautiful R code and helped me immensely with statistics.

My little office was filled with massive love and support for one another: Linda, Janine, Paola, and Andreia. You put up with my moaning about worm sperm so many times and my spontaneous statistic-based weeps – you all deserve medals.

The rest of the department were awesome as well. Every single one of you deserves a mention for your kindness and encouragement and for the times when things got a bit tough: Dan Ingram, Claire Webster, Lena Grinstead, Paul Parsons, Dave Treanor, Claudia Gray, Beth Gibson, Julia Jones, Jenny James, Tanya Pennell, Kat Lund-Hansen, Chris Sandom, Rosaline Hulse, Joanne Carnell, Maria Clara Castellanos, Alan Stewart, Steve Pearce, Martyn Stenning and Jeremy Niven.

I have to say an extra special thank you to Beth, Claire, Ellie, Cristina, Dan, Linda and Janine. You are my dream team. I would also like to thank my family (especially my amazing sister Anna) who offered encouragement and advice. Also to my dear friends Katie Savíc, Darya Stramentov and Maria Duignan.

And finally, the man who deserves an honorary PhD for the years of love and support he has given me – John Lloyd. Thank you for the hours you spent shifting through shite, building cages, worm wrangling, feeding me wine, measuring, running round fields, catching flies, and keeping the faith when my brain turned to blubber. I quite simply couldn't have done it without you.

And thank you Norman the cat. And thank you vitamin G&T.

# **Chapter 1** - General introduction

### 1.1 Decline in farmland biodiversity

Agriculture represents the dominant land use throughout much of Western Europe, and a significant part of European biodiversity is associated with this habitat (Robinson & Sutherland, 2002). In 2002, at the World Summit on Sustainable Development, 190 countries committed themselves to achieving a significant reduction to the current rate of biodiversity loss at global, regional and national scales (Balmford *et al.*, 2005). By 2010 it became clear that the conservation efforts and practices put into place to achieve this target had failed, whilst indicators of pressures on biodiversity had simultaneously increased (Butchart *et al*., 2010). Currently, of all the drivers of biodiversity loss – including climate change and urbanisation – it is believed that the intensive management of agricultural land over the past c80 years has had the largest overwhelmingly negative impact on wildlife (Hayhow *et al*., 2016).

The UK has a strong tradition of natural history and much of the long-term information about species' populations come from volunteer naturalists, who have collected these data for over a century. Birds in particular are considered to be one of the best monitored taxonomic groups in the world (Eaton *et al*., 2015), and for this reason bird data are often used as indicators of the effects of environmental change. The combined population index for all species of native breeding wild bird populations in the UK sits 7% below its 1970 value (DEFRA, 2017a), providing strong evidence for the overall decline in biodiversity.

However, when this index is divided into habitat groups (Figure 1.1), farmland birds have fared particularly badly compared to species from other habitats over the same period. Between 1970 and 2016, the unsmoothed index for farmland specialists declined by 70% (the smoothed trend is also a 70% decline), while for farmland generalists it declined by 12% (13% in the smoothed trend) (DEFRA 2017a). This marked decline has been attributed to the rapid changes in farmland management that occurred during this period (DEFRA 2017a). Although the rate of farmland bird decline has slowed in recent decades it still continues, with the smoothed index between 2010 and 2016 showing a 10% drop (DEFRA, 2017a). This sensitivity to the effects of agricultural intensification (Butchart *et al*., 2010; Wilson *et al.*, 2010) makes farmland bird populations a sensitive proxy for wider biodiversity health on farmland (Butler *et al*. 2010).

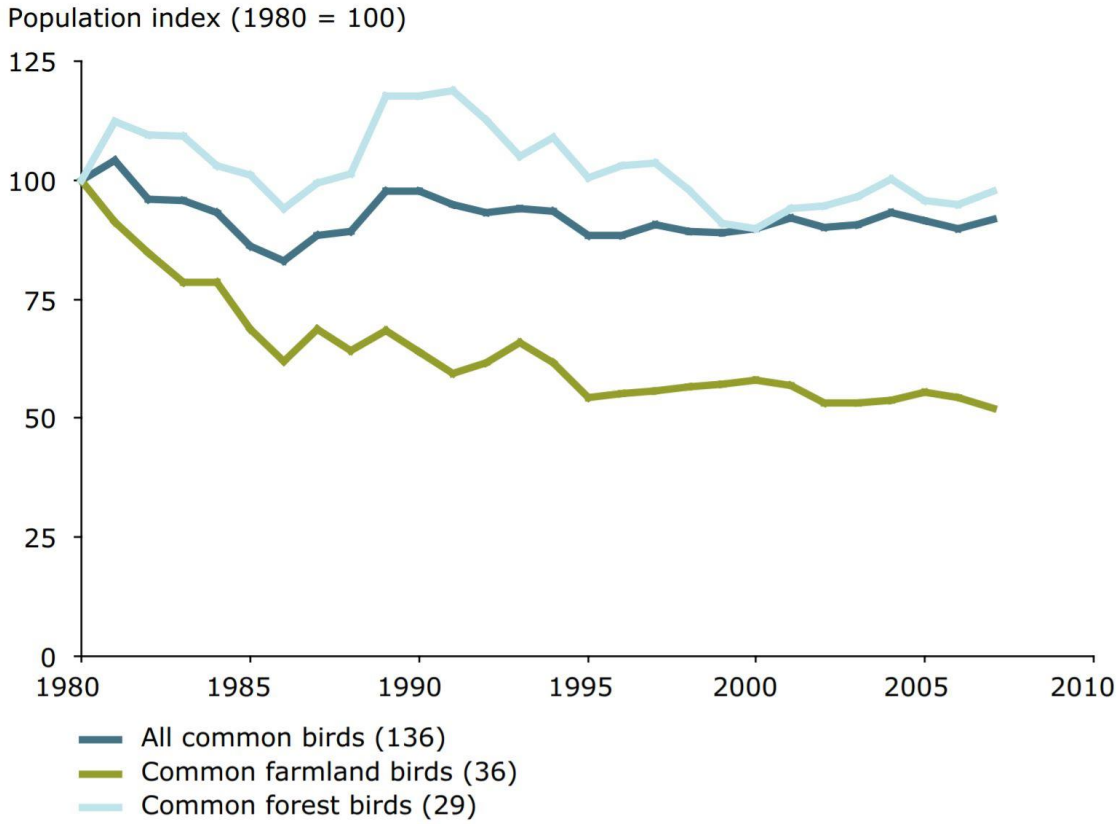



**Figure 1.1** Common birds (total, farmland and forest) in Europe population index. Figure reproduced from EEA, 2010.

Although birds are the best-studied wildlife taxa, evidence also suggests that other species are in decline. For example, the 'State of Nature' report published in 2016 by more than 50 conservation bodies found that insects and other invertebrates that make up 97% of all animal species were particularly struggling, with 59% in decline since 1970 (Hayhow *et al*., 2016).

## 1.2 Agricultural intensification and the use of chemicals

The management of terrestrial ecosystems to focus on human needs has affected all aspects of the agroecosystem (Chamberlain *et al*., 2000; Firbank *et al*., 2008). Since 1945, there has been a 65% decline in the number of UK farms. Individual farms have become ever more specialised with larger fields, operating more efficiently, and generating almost a four-fold increase in yield (Robinson & Sutherland, 2002). However, the corresponding changes in farming practices – including a loss of mixed farming; changes in grassland management; the removal of non-cropped farmland features like hedgerows and ponds; and a move from spring to autumn

sowing of arable crops, resulting in a loss of winter stubble – have had negative effects on farmland bird populations (Chamberlain *et al*., 2000; Robinson & Sutherland, 2002).

One notable change in farmland management associated with such intensification is an increase in the use of agrochemicals. In order to meet the food demands of a rising global population, the use of agrochemicals in the 'Green Revolution' has allowed us to break what was once the tight local recycling of nutrients on individual farms (Tilman, 1998), reducing the need for rotational farming and the requirement for animal manure through the application of inorganic fertilisers (Robinson & Sutherland, 2002); and to protect crops with new pesticides. Farming practices have thus become ever more dependent on the industrial scale input and application of chemicals (Figure 1.2). Up until the early 1940s, pest control was achieved with applications of botanicals and inorganic compounds including pyrethrum, rotenone and nicotine (Casida & Durkin, 2013). Dichlorodiphenyltrichloroethane (DDT) was first used as a potent chemical insecticide in 1948, and was used effectively and extensively worldwide. However, by 1972 it was identified as being dangerous to mammals, birds and other organisms, and most uses were eventually banned (Oberemok *et al*., 2015). DDT and its chemical relatives were replaced by the widespread use of organophosphates and carbamates to control sucking insects on crops and fill other gaps in pest management protocols (Casida & Durkin, 2013; Oberemok *et al*., 2015). These are still widely used preparations (19% of the world market), despite the evidence of harm to the environment (Casida & Durkin, 2013).

A response to the increasing reliance on synthetic agrochemicals of conventional farming is the rise of the Organic farming movement, believed to use more environmentally sound methods of pest control. It is also argued that organic farming methods generally increase biodiversity, reversing declines that have resulted from other modern agricultural practices (Bengstonn *et al.* 2005). The ecological benefits of organic versus conventional farming are the subject of debate – do the oft decreased yields from organic farms negate the local benefits to biodiversity that the methods deliver? (Tuck *et al.* 2014). The answer appears to be yes. A hierarchical meta-analysis of land-use intensity and the effects of organic farming on biodiversity found that on average, organic farming increased species richness by 30% (this result has been robust over the last 30 years and shows no signs of diminishing) (Tuck *et al.* 2014). Tuck *et al.* confirmed that organic farming has large positive effects on biodiversity compared to conventional farming, but that effect size varied with the organism and crop group studied.

Currently, in the European Union, there are approximately 500 pesticides authorised for use, including insecticides, herbicides, fungicides and growth regulators (Milner & Boyd, 2017), and the most widely used of these insecticides globally are neonicotinoids (Jeschke & Nauen, 2008; Jeschke *et al*., 2011; Oberemok *et al*., 2015; Wood & Goulson, 2017).

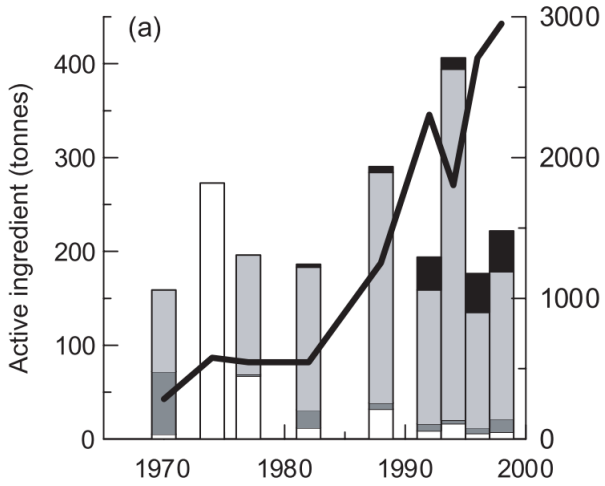



**Figure 1.2** The increasing inputs in farming. (a) Area sprayed with insecticide in England and Wales (line and right axis) and amount (active ingredient) of each type of insecticide used (bars and left axis): carbamates (white), organo-chlorines (dark grey), organo-phosphates (light grey) and pyrethroids (black), this breakdown not available for 1974. Note: Active ingredient potency has increased over time which can mean less weight of active ingredient applied. Reproduced from Robinson and Sutherland 2002.

### 1.3 Introduction to neonicotinoids

The introduction of neonicotinoids in early 1990 was considered a breakthrough in the agrochemical sector (Jeschke & Nauen, 2008), and they now comprise approximately 30% by value of the global insecticide market, registered in more than 120 countries (Jeschke *et al*., 2011; Godfray *et al*., 2015). Designed to replace an older, more damaging generation of pesticides, the selectivity of neonicotinoid compounds to insects (and their targeted application methods) meant that they were initially regarded as more efficient and generally safer for the environment. In 1990, before the launch of the first neonicotinoid imidacloprid, the market was dominated by organophosphates, pyrethroids and carbamates (market share: 43%, 18% and 16% respectively; by 2005, neonicotinoids had already started to replace organophosphates (25%) and carbamates (10%)) (Figure 1.3) (Elbert *et al*., 2008).

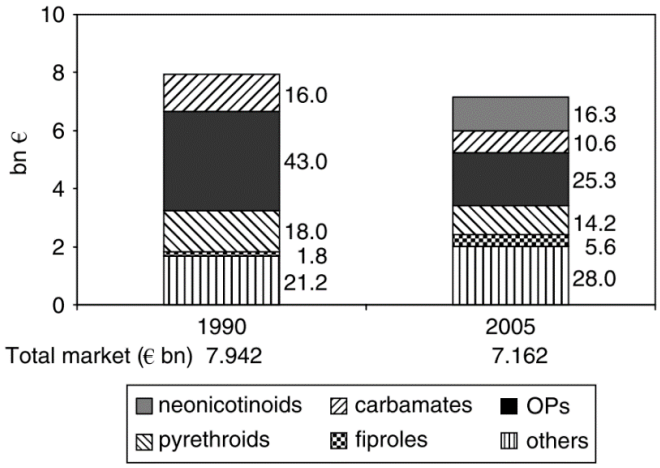



**Figure 1.3** Development of insecticidal classes in crop protection, 1990–2005, expressed as percentage of total. Reproduced from Elbert *et al*. (2008).

The UK has detailed data that chart the widespread adoption of neonicotinoids, compared to other typical insecticides (Figure 1.4) (DEFRA, 2017b). Imidacloprid quickly dominated the insecticide market when it was first introduced in to the UK in 1994. Although its use on arable crops has declined, it is now available off-patent and this has led to the broad scale use of this compound in numerous products for use as insecticides in the domestic garden (Elbert *et al*., 2008). Thiamethoxam was launched in 1998 by Syngenta, and as of 2008 held licences for 115 crop uses in at least 64 countries on a wide range of crops including cereals, potatoes and fruit (Elbert *et al*., 2008). Clothianidin (a metabolite of thiamethoxam, owned by Bayer) was launched in 2002 and can be used on a similar range of targets to imidacloprid, including corn and oilseed rape, with registrations for use on 40 other crops (Elbert *et al*., 2008).

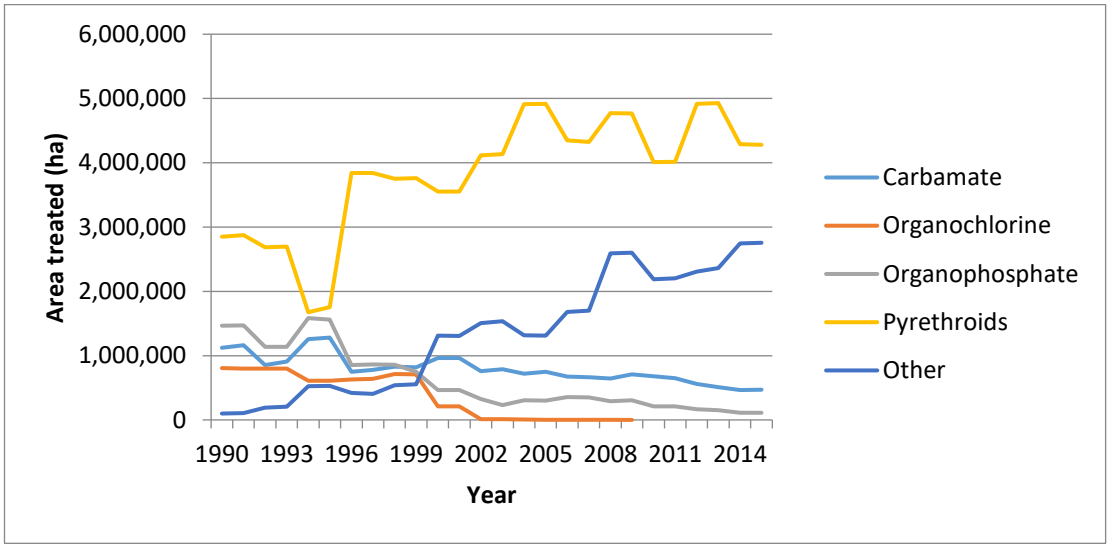



**Figure 1.4** Area treated (ha) of carbamates, organochlorines, organophosphates, pyrethroids and other pesticides (including neonicotinoids) from 1990 to 2015. Area treated refers to the active substance treated area. This is the basic area treated by each active substance, multiplied by the number of times the area was treated (DEFRA, 2017b)

### *1.3.1 Mode of action*

Neonicotinoids are neuroactive insecticides which target nicotinic acetylcholine receptors in the insect nervous system, causing nervous stimulation at low concentrations, and paralysis and death at higher concentrations (Goulson, 2013). Their widespread adoption is attributable to their flexibility of use (Jeschke *et al*., 2011): they can be applied as foliar sprays, soil drenches, or incorporated in granular form, but are most commonly used as seed dressings (60% of all applications (Jeschke *et al*., 2011)). They are applied to a wide variety of crops including cotton, fruit, rice, vegetables, cereals and rape (Goulson, 2013, Elbert *et al*., 2008).

Thiamethoxam is one of the most commonly used pesticides from the neonicotinoid group (Simon-Delso *et al*., 2015) and has high water solubility (average DT50 = 229 days, 4,100 mg/L), which means it is persistent in the environment with high potential to be transported into surface water via run-off or groundwater discharge (Main *et al*., 2014). Thiamethoxam is metabolised to clothianidin in insects, other animals, plants and soil (Simon-Delso *et al*., 2015). Clothianidin is currently the most commonly used seed treatment worldwide and also displays high water solubility (Simon-Delso *et al*., 2015). As water-soluble systemic pesticides, neonicotinoids become incorporated into all parts of the plant tissue as the plant grows, and it is this systemic mode of action that provides the effective and consistent control of aphids, leafhoppers, coleopteran and lepidopteran pest species (Elbert *et al*., 2008). However, it is also their systemic nature which can result in contamination of pollen and nectar, posing an exposure risk to pollinators (Rortais *et al*., 2005; Bonmatin *et al*., 2015; Botías *et al*., 2017; Woodcock *et al*., 2018).

### *1.3.2 Risk to pollinators*

In recent years, numerous studies have raised concerns about the impact of neonicotinoids on non-target pollinators. The majority of these studies have focused on the honeybee and bumblebees, due in part to the fact they are reared commercially for pollination (Lundin *et al*., 2015; Wood & Goulson, 2017). Laboratory and field studies suggest that exposure to field-relevant doses of neonicotinoids (the doses typically found in the nectar and pollen of flowering crops) can impair pollen collection, increase worker mortality, reduce the production of new queens, weaken the bee's immune system, and affect the weight of honeybee queens (Gill *et al*., 2012; Whitehorn *et al*., 2012; Di Prisco *et al*., 2013; Gill & Raine, 2014). It is now established that neonicotinoids cause sub-lethal effects at field-realistic doses; furthermore,

two large field trials have found negative effects in field-realistic scenarios (Rundlöf *et al*., 2015; Woodcock *et al*., 2017).

In response to this mounting evidence the European Commission mandated the European Food Safety Authority (EFSA) to produce risk assessments for the use of clothianidin, imidacloprid and thiamethoxam and their impact on bees (EFSA 2013a, b, c). Assessments concluded that use of these chemicals on flowering crops posed a significant and unacceptable risk to bees (EFSA 2013a, b, c). The evidence presented to the EU Member States focussed on bee health, without detailed consideration of the risks posed by contamination of the wider environment, or to other non-target organisms. In 2013, the European Union voted for a temporary 2-year moratorium on the application of those three neonicotinoids (via seed treatments) on mass-flowering crops deemed to be attractive to bees (e.g. oilseed rape and sunflowers) (EU Commission 2013). This move was strongly opposed by many in the farming community and vigorous debate ensued, focusing on the evidence of harm to pollinators by neonicotinoids, and the economic and environmental costs and benefits of the restrictions (Godfray *et al*., 2015).

In 2015, the European Commission asked EFSA to re-evaluate the evidence. Following a two-year review of over 1,500 studies, on 28th February 2018 EFSA published a series of reports concluding that most uses of neonicotinoids represent a risk to wild bees (EFSA, 2018). Unlike the previous assessment, this work included bumblebees and solitary bees, both of which were deemed to be at risk. Thus, at the time of writing, the moratorium is still in place and the European Union is considering extension to cover other uses of neonicotinoids (which will be discussed further in Chapter 7).[1]

[1] In late April 2018, EU member states voted in favour of an almost complete ban on the use of neonicotinoid pesticides across the EU. This move represented a major extension of existing restrictions put in place in 2013. In 2013, a partial ban was implemented by the EU on the use of three chemicals in the neonicotinoid class: thiamethoxam, clothianidin and imidacloprid. Use was restricted to crops including maize, wheat, barley, oats and oilseed rape. However, the new regulation goes much further to include a ban on all outdoor uses of the chemicals. This action has been driven by a recent report from the European Food Safety Authority (EFSA) which found that neonicotinoids posed a threat to many species of bees (discussed further in Chapter 7). Currently, neonicotinoids will only be able to be used in greenhouse conditions across the EU.

## 1.4 Wider environmental contamination

The majority of research to date has focussed on the hazard posed to bees from applying neonicotinoids to flowering crops. However, there are growing concerns about the potential contamination of semi-natural habitats on farmland, and of the wider environment, with neonicotinoids. For example, a recent study has shown that honey samples from hives in the UK are still found to contain traceable levels of neonicotinoids, despite the EU moratorium being in place since autumn 2013 (Woodcock *et al*., 2018). The contamination of honey by neonicotinoids is a global problem: 75% of honey sampled across the globe were found to contain at least one of five tested neonicotinoid compounds (Mitchell *et al*., 2017). It has emerged that neonicotinoid contamination is a risk for non-target vegetation including wildflowers, and also soil and aquatic systems.

### *1.4.1 Neonicotinoids in soil*

Studies suggest that the majority of neonicotinoid used in seed treatments is lost to the soil, with between 1.6% and 20% of the active ingredient is taken up by the developing crop, some is lost as dust whilst drilling (<2%), leaving the remaining ~90% in the soil (Sur & Stork, 2003; Tapparo *et al*., 2012; Goulson, 2013). Since neonicotinoids are highly water-soluble, these leftovers enter the soil water. The time that they remain active depends on the soil type, and characteristics of the individual compound (Goulson, 2013). Neonicotinoids can also bind to soil particles, and the strength at which they bind influences the rate at which they leach out of soil if water is present (Goulson, 2013). The dissipation half-life times (DT50's) of neonicotinoids in soil are highly variable and typically range from 28 - 1250 days for imidacloprid, 7 - 3001 days for thiamethoxam, and 148 - 6931 days for clothianidin (Goulson, 2013). Additional factors such as pH can also affect degradation: for example, thiamethoxam persists longer in acidic soils than alkaline (Karmakar & Kulshrestha, 2009).

Recent research has looked at neonicotinoid concentrations in agricultural soils, under field realistic scenarios, and measured their potential to accumulate through field trials and sampling (Table 1.1). Work by Jones *et al*. (2014) investigated neonicotinoid levels of 18 arable soils in six different locations across the UK. Residues in the centre of fields were found to be higher than those around the perimeter; soil samples taken from the centre of fields contained neonicotinoid concentrations ranging from 0.02 to 13.6 ppb, thiamethoxam concentrations were between <0.02 and 1.5 ppb, and imidacloprid concentrations were between <0.09 and 10.7 ppb (Jones *et al*., 2014). Imidacloprid had not been used in the three years that preceded

the study, so its detection was either as a result of contamination by dust drift or was more likely due to persistent residues from previous applications (Jones *et al*., 2014).

Botías *et al*. (2015) analysed soil samples from five winter-sown wheat fields and seven winter sown oilseed rape fields, collected in the spring of 2013, 10 months after the crop was sown. Samples were also collected from the centre and edge of the field, and like Jones *et al.* (2014), residues from the centre of the field were higher than those of the field edges (imidacloprid: range ≤0.07–7.90 ppb, average 3.03 ppb, field edge 1.92 ppb; thiamethoxam: range ≤0.04–9.75 ppb, average 3.46 ppb, field edge 0.72 ppb; clothianidin: range 0.41–28.6 ppb, average 13.28 ppb, field edge 6.57 ppb) (Botías *et al*. 2015).

These research outputs demonstrate that, due to their long half-lives and potential for accumulation (Goulson, 2013), detectable levels of neonicotinoids are present in the soil for longer than the average agricultural cycle. Thus that most soil-dwelling organisms in conventional arable farmland are likely to be chronically exposed to fluctuating concentrations of neonicotinoids (Wood and Goulson, 2017; Goulson, 2013). Evidence of bioaccumulation of neonicotinoids following long-term exposure has been shown in the earthworm *E. andrei* (Chevillot *et al.* 2017), and this bioaccumulation was concomitant with a significant increase in DNA damage (as measured by comet assay) and significant effects on reproduction. This represents an additional potential point of entry of neonicotinoids into the wildlife food chain (Chevillot *et al.* 2017).

### *1.4.2 Neonicotinoids in water*

Surface waters have been found to be contaminated by neonicotinoids, including puddles, ditches, irrigation channels and streams in or near farmland, (Van Dijk *et al*., 2013; Main *et al*., 2014; Samson-Robert *et al*., 2014; Morrissey *et al*., 2015; Schaafsma *et al*., 2015). Waterbodies can be contaminated by a variety of routes, including by leaching into groundwater, via the decay of treated plant material, by contaminated dust from the drilling of seed, or by spray drift (Wood and Goulson, 2017). Due to the persistence of both thiamethoxam and clothianidin in soil and their high water solubility, there is serious potential for unintended transport into surface waters.

Contamination levels of various types of surface waters differ. For example, samples taken from within and around the perimeter of corn fields in Southwest Ontario detected residues of

clothianidin (mean = 2.28 ppb, maximum = 43.60 ppb) and thiamethoxam (mean = 1.12 ppb, maximum = 16.50 ppb) in 100% and 98.7% of samples tested, respectively (Morrissey *et al*., 2015; Schaafsma *et al*., 2015). Streams near to fields of corn and soybean production in the USA contained median levels of 8.2 ppb of clothianidin and levels of <2ppb thiamethoxam (Hladik, *et al*., 2014).

A recent report by Buglife (a UK invertebrate conservation charity) investigated the pollution levels of 26 sites around the UK for five of the most commonly-used neonicotinoids (Imidacloprid, Clothianidin, Thiamethoxam, Acetamiprid and Thiacloprid) using results from the European Union's Water Framework Directive watchlist (Shardlow, 2017). Seventy four percent of the reported sites contained neonicotinoids; eight rivers exceeded the chronic pollution limit, and two rivers exceeded the acute pollution limit (below 0.2 ppb (short-term acute) or 0.035 ppb (long- term chronic) (Morrissey *et al*. 2015)), indicating the UK has a significant problem with neonicotinoid contamination of freshwater.

This is an international problem. Imidacloprid – one of the earlier and most widely used neonicotinoids – has been found in the Netherlands in groundwater, streams and ditches at concentrations far exceeding the maximum allowable risk level (13 ng/L) (Van Dijk *et al*., 2013). It has also been detected in 89% of rivers, creeks and drains in California, with 19% of those samples exceeding the US Environmental Protection Agency's chronic invertebrate Aquatic Life Benchmark guideline of 1.05 ppb (Starner & Goh, 2012). However, a comprehensive review of water monitoring literature suggests that levels of clothianidin and thiamethoxam are often well below 1 ppb (0.003 to 3.1 ppb for clothianidin, and 0.001 to 225 ppb for thiamethoxam) (Morrissey *et al*., 2015).

**Table 1.1** Summary of studies published since 2013 that document neonicotinoid concentrations in agricultural soils (Reproduced from Wood and Goulson, 2017).

| Sample size (fields) | Country | Year(s) studied | Samples collected | Previously cropped | Mean neonicotinoid concentration (ng/g) | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | | Imidacloprid | Clothianidin | Thiamethoxam | |
| 28 | USA | 2012 | Spring, pre-planting | Various | 4.0 | 3.4 | 2.3 | Stewart *et al.* (2014) |
| 18 | UK | 2013 | Spring | Various | 1.62 | 4.89 | 0.4 | Jones *et al.* (2014) |
| 25 | Canada | 2013 and 2014 | Spring, pre-planting | Maize | | 3.45 | 0.91 | Limay-Rios *et al.* (2015) |
| 7 | UK | 2013 | Summer, with crop (10 months post planting) | Oilseed rape | 3.03 | 13.28 | 3.46 | Botías *et al.* (2015) |
| 3 | USA | 2011 to 2013 | Continuously | Maize and soybean | | 2.0–11.2 | | de Perre *et al.* (2015) |
| 50 | USA | 2012 and 2013 | Summer, with crop | Maize | | 7.0 | | Xu *et al.* (2016) |
| 27 | Canada | 2012 to 2014 | Summer, with crop | Oilseed rape | | 5.7 | | Xu *et al.* (2016) |
| 35 | Germany | 2013 | Autumn, pre planting | Various | | 2.1 | | Heimbach *et al.* (2016) |

**Table 1.2** Summary of studies published since 2013 that document mean neonicotinoid residues in wild plant tissues, pollen and nectar in plants growing close to neonicotinoid-treated agricultural crops (Reproduced from Wood and Goulson, 2017).

| Sample size | Vegetation adjacent to: | Samples collected | Sample type | Mean neonicotinoid concentration (ng/g) | | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | Thiamethoxam | Clothianidin | Imidacloprid | Thiacloprid | |
| 43 | Oilseed rape | May–June 2013 | Pollen | 14.81 | | 0.56 | <0.04 | Botías *et al.* (2015) |
| 55 | Wheat | May–June 2013 | Pollen | 0.14 | | <0.16 | <0.04 | Botías *et al.* (2015) |
| 24 | Oilseed rape | May–June 2013 | Nectar | 0.10 | | | | Botías *et al.* (2015) |
| 8 | Wheat | May–June 2013 | Nectar | <0.10 | | | | Botías *et al.* (2015) |
| 33 | Maize | Summer 2014 & 2015 | Nectar [a] | | 0.2–1.5 | | | Mogren and Lundgren (2016) |
| 40 | Maize | June 2014 | Foliage | | 0.4 | | | Pecenka and Lundgren (2015) |
| 50 | Maize | July 2014 (1 month after planting) | Foliage | | 0.69 | | | Pecenka and Lundgren (2015) |
| 100 | Oilseed rape | May–June 2013 | Foliage | 8.71 | 0.51 | 1.19 | | Botías *et al.* (2016) |
| 375 | Maize | Summer 2014 & 2015 | Foliage | | 0.5–13.5 [b] | | | Mogren and Lundgren (2016) |
| 6 | Maize | Summer 2011 | Complete flower | 1.15 | 3.75 | | | Krupke *et al.* (2012) |
| 78 | Various | Summer 2012 | Complete flower | 7.2 | 1.4 | 1.1 | | Stewart *et al.* (2014) |
| 7 | Oilseed rape | April–May 2013 (2 days after sowing) | Complete flowers and foliage | | 1.2 | | | Rundlöf *et al.* (2015) |
| 8 | Oilseed rape | April–June 2013 (2 weeks after sowing) | Complete flowers and foliage | | 1.0 | | | Rundlöf *et al.* (2015) |

The results of Krupke *et al.* (2012) are included for reference

[a] Mogren and Lundgren (2016) sampled honeybees foraging on wild plants and directly extracted nectar from their crop. See main body of text for further discussion

[b] Range of concentrations, data on mean concentrations not available

The risks posed by contamination of surface waters is dependent on the persistence of the compound in that environment. Studies investigating the breakdown of imidacloprid, thiamethoxam and clothianidin show DT50 ranges lasting minutes to several weeks in water, depending on the processes involved (i.e. temperature, pH, hours of daylight) (Anderson *et al*., 2015). A photolysis study by Peña *et al*. (2011) found thiamethoxam was susceptible to photolysis, but that the presence of suspended particulate matter and dissolved organic carbon affected photodegradation rates. The DT50 for clothianidin in water is less than half a day via photolysis, but in situations where there is little exposure of the water to natural light, degradation is likely to be much slower, with a potential to accumulate over time (Anderson *et al*., 2015).

### *1.4.3 Neonicotinoids in field margin and non-crop plants*

The current prophylactic use of neonicotinoids as seed dressings on many arable crops (Lundin *et al*., 2015), combined with their persistence, solubility in water and systemic action in plants, presents a large-scale risk of neonicotinoid contamination of non-target plants. A number of recent studies report the presence of neonicotinoids in the pollen, nectar, and leaves of non-target vegetation (Table 1.2).

#### Pollen and nectar

Botías *et al*. (2015) sampled pollen and nectar from wildflower species found in the field margins of winter wheat and oilseed rape fields in the UK. Overall, the pollen from wildflowers contained higher levels of neonicotinoid residues than pollen from the treated crop, although there was also substantial variation in the levels of residues found in the same species of wildflower. Average levels of total neonicotinoid contamination in wildflower pollen were significantly higher in margins adjacent to treated oilseed rape (~15 ng/g) than for margins adjacent to treated wheat (~0.3 ng/g) (Botías *et al*., 2015).

Residues found in nectar were much lower than those in pollen; only thiamethoxam was detected at average levels of 0.1 ppb in wild flowers adjacent to oilseed rape fields and <0.1 ppb adjacent to wheat fields (Botías *et al*., 2015). Mogren and Lundgren (2016) tested the residues of nectar concentrations by sampling the content of honeybee crops. Clothianidin levels of 0.2 and 1.5 ppb were found, with significant differences found between wild plant

species (Mogren & Lundgren, 2016). An earlier study by Krupke *et al*. (2012) found dandelions sampled near fields of maize contained up to 9.4 ppb clothianidin and 2.9 ppb thiamethoxam.

### Foliage

In a recent review of the post-2013 evidence for environmental risks posed by neonicotinoids, Wood and Goulson (2017) suggest that exposure originating from contaminated non-target plants poses a "greater risk" than had been appreciated prior to 2013. One area of particular concern is the uptake of neonicotinoids by non-crop plants, and the subsequent contamination of their foliage. The contamination of wild plants has been investigated in the most comprehensive study to date by Botías *et al*. (2016). Samples were taken from forty-five species of wild plants growing adjacent to treated oilseed rape crops. Neonicotinoid levels found in field margin plants ranged from ≤0.02 to 106 ng/g, with the average total neonicotinoid contamination being 10 ng/g. These high levels of variation were also found by Mogren and Lundgren (2016) in the foliage of seven wildflower species, with clothianidin levels ranging from 0 - 33 ppb in phacelia to 0 - 81 ppb in sunflowers. Pecenka and Lundgren (2015) sampled for clothianidin levels in milkweed *Asclepias syriaca* growing alongside treated maize; mean levels of 0.58 ng/g were found, with a maximum of 4.02 ng/g.

## 1.5 Impacts on non-target organisms

Comprehensive reviews exist investigating the impact of neonicotinoids on some non-target organisms. Goulson (2013) provided the first overview of the environmental risks posed by neonicotinoids followed by Pisa *et al*. (2015) who outlined an extensive review of the literature, describing the effects of neonicotinoids on non-target invertebrates. Gibbons *et al*. (2015) reviewed the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife, and Wood and Goulson (2017) looked specifically at the body of evidence post-2013. Due to the sheer volume of published data on neonicotinoids over the last decade, the most recent reviews (Giorio *et al*., 2017; Pisa *et al*., 2017; Furlan *et al*., 2018) focus on gaps in knowledge that have been addressed after publication of the Worldwide Integrated Assessment (WIA) on systemic insecticides in 2015. For the purposes of this review I will focus on the organisms I have studied in this body of work. Each section relates to a chapter in this thesis which I will summarise at the end of each section.

### *1.5.1 Aquatic invertebrates*

The most comprehensive review on the impact of neonicotinoid contamination of surface waters and its associated risks to aquatic species was undertaken by Morrissey *et al*. (2015). Over 200 studies were reviewed for six neonicotinoids, with 49 different aquatic arthropod species, spanning 12 orders. The sensitivities (for both acute and chronic toxicity) varied greatly among aquatic arthropods, with species belonging to the class Insecta being typically the most sensitive; the Ephemeroptera, Trichoptera and several Diptera, particularly the Chironomidae (midges), were consistently the most sensitive taxa (Morrissey *et al*. 2015).

The most widely tested species *Daphnia magna* (the Cladoceran water flea) is the global standard for commercial toxicity tests (Sánchez-Bayo, 2006) and represents 16% of the toxicity tests in the Morrissey *et al*. (2015) review. Though *D. magna* has a wide range of sensitivity to neonicotinoids, it is also reported to be the least sensitive test species for acute and chronic neonicotinoid studies by far. The short-term L[E]C50 is at least two to three orders of magnitude higher than the geometric means for most other aquatic invertebrate species (Morrissey *et al*., 2015). Clothianidin has been found to have no lethal or sub-lethal effects on *D. magna* at concentrations over 100 ppb, whereas *Chironomus dilutus* exhibited EC50 effects at 1.85 ppb and LC50 effects at 2.32 ppb (de Perre *et al.*, 2015). This strengthens the argument for the use of different model organisms, since relying on the relatively insensitive *D. magna* may not be a reliable benchmark for other species (Beketov & Liess, 2008).

Of the 214 studies reviewed by Morrissey *et al*. (2015), imidacloprid was by far the most commonly studied neonicotinoid (66% of studies). Van Dijk *et al.* (2013) found a significant negative relationship between imidacloprid polluted surface water and macro-invertebrate abundance, with macro-fauna abundance dropping off sharply between 0.013ppb and 0.067ppb imidacloprid; concentrations more than an order of magnitude below the Environmental Protection Agency guidelines. As we have seen, imidacloprid is now relatively little used in the UK, being largely replaced by thiamethoxam and clothianidin (Goulson, 2013). The results of an extensive review of laboratory and semi-field microcosm studies indicates that aquatic invertebrates are highly sensitive to neonicotinoids (Pisa *et al*., 2015), and there is a need to investigate the effects of the newer neonicotinoids on aquatic ecosystems, at field-realistic doses. Chapter 4 describes an experiment that tests the effect of field-realistic doses of clothianidin and thiamethoxam on the colonisation and development of aquatic invertebrate populations, in puddle-replicate microcosms under semi-field conditions.

### *1.5.2 Worms*

The application of agricultural products for the protection of crops has been shown to introduce these compounds to the drilosphere (the part of the soil which is influenced by earthworm secretions and castings), where the soil acts as a sink for agrochemicals (Givaudan *et al*., 2014). Earthworms are highly likely to be chronically exposed to fluctuating levels of neonicotinoids in the soil, either via direct consumption of contaminated plant material and soil, or through direct contact with contaminated soil, water or treated seeds (Goulson, 2013). Earthworms have the same neural pathways that neonicotinoids target in pest species (Volkov *et al*., 2007) and so the presence of neonicotinoids in soil could potentially affect the vital role of earthworms in the environment. Pisa *et al*. (2015) conducted an extensive recent review of the impacts of neonicotinoids at varied concentrations on survival, reproduction and behaviour across different earthworm species. They concluded that individuals are at risk of mortality if they consume soil or organic particles of about 1 ppm for several days. However, very few data are available for field realistic neonicotinoid exposure of ecologically relevant earthworm species. The majority of experiments use the compost worm *Eisenia fetida* with little consideration given to the sensitivities of other species. *E. fetida* are not typically found in areas where neonicotinoids are in use, preferring warm, moist habitats, with a ready supply of fresh compost material. Further, they are claimed to be less sensitive to environmental toxicants than other earthworm species (Dittbrenner *et al*., 2010) and so give little indication of the potential impacts of pesticides in arable systems.

Anecic worms like *Lumbricus terrestris* feed on the surface of the soil, dragging food into their wide and deep-penetrating burrows (Nuutinen *et al.*, 2011). They do not respond well to physical disturbance from tillage and so are typically found in undisturbed field margins which may act as source areas for further populations (Nuutinen *et al.*, 2011). Very few studies to date have investigated the impact of neonicotinoids on *L. terrestris*, but those that have, focus on the neonicotinoid imidacloprid. Imidacloprid exposure produced little or no measurable impact on survival at 4 ppm (Dittbrenner *et al*., 2012), but resulted in a moderate decrease in body mass and a large decrease in cast production being observed at 0.66 ppm (Dittbrenner *et al*., 2010). Cast production was negatively affected at 0.66 ppm and 0.189 ppm (Capowiez *et al*., 2010).

A study of agrochemical toxicity to *E. fetida* found clothianidin to be the most toxic of 45 pesticides tested (Wang *et al*., 2012). Since clothianidin is becoming the most commonly used

neonicotinoid in the UK (DEFRA, 2016), and persists in soil, the study described in Chapter 3 of this thesis focuses on clothianidin. The study investigated the effects of field-realistic doses of clothianidin on mortality, weight gain, and food consumption of *L. terrestris* individuals, to assess the fitness impact of chronic clothianidin exposure.

### *1.5.3 Hoverflies*

As discussed above, surface waters in and around farmland are at risk of contamination by neonicotinoids and even low levels of neonicotinoids have been shown to have negative effects on aquatic invertebrates. Adult hoverflies (Syrphidae) perform an important pollination role, often considered to be the second most important pollinator after bees (Larson *et al.*, 2001), yet a comprehensive review by Wood & Goulson (2017) found no post 2013 research on the effects of neonicotinoids on either adult or larval hoverflies; nor was there any research prior to 2013. Hoverfly larvae have specialist beneficial roles in the agroecosystem, different to hoverfly adults; some are valued biocontrol agents as they are aphidophagous, whilst others are saprophagous (Gilbert *et al*., 1994) and play an essential part in the decomposition process by breaking up and aerating compost, dung, and dead wood (Gilbert 1985).

*Eristalis tenax* is one of the most common species of hoverfly in the UK (Ball and Morris, 2013), has pollination value in open and closed crop production systems, and at high densities has a pollination effort comparable to the efficacy of small honeybee colonies (Jauker *et al*., 2012). It is for these reasons that *E. tenax* was used as a study species in this thesis. Chapter 2 describes the impacts of chronic exposure of thiamethoxam on the development of *E. tenax* larvae, and latent effects on behaviour in the adult fly.

### *1.5.4 Butterflies*

As with bees, the cause of butterfly losses are the subject of debate. UK farmland population declines have accelerated since the mid 1990's, despite significant investment in agri-environment schemes (Fox *et al*., 2015; Gilburn *et al*., 2015). Two recent correlational studies, one in the UK (Gilburn *et al*., 2015) and one in the USA (Forister *et al*., 2016) found there to be a significant correlation between neonicotinoid usage and the rate of butterfly decline. However, as with all correlational studies, it is often hard to disentangle the effects of other farming practices that may be having a negative impact on butterfly populations (Goulson & Nicholls, 2016). Most of the studies investigating the effect of neonicotinoids on butterflies and moths have been conducted on pest species (spanning 32 species of moths from nine

families) (Pisa *et al*., 2015). There are variations in reported sensitivity between species, with some moth species being up to 100 times more sensitive than others (Stará & Kocourek, 2007). Due to an overall lack of toxicity data for wild, non-pest Lepidopteran species, it is important to investigate this group's sensitivity to neonicotinoids.

The Common Blue butterfly (*Polyommatus icarus)* is the most widespread blue butterfly, on UK farmland, and has undergone a 17% long term (1976 – 2014) decline in abundance and occurrence (Fox *et al*., 2015); furthermore, between 2000 and 2009 there was a 30% decline in its 10-year population trend (Gilburn *et al*., 2015). Due to the prevalence of neonicotinoid contamination in field margin plants (Botías *et al*., 2015), species like *P. icarus* are at risk of consuming contaminated vegetation during their larval stage and neonicotinoids present in nectar during their adult stage. The studies described in Chapter 5 focus on larval exposure; first establishing typical vegetative contamination levels of *P. icarus'* food plants in the field, and then experimentally testing the effect of field-realistic doses of clothianidin via oral exposure on the mortality and development of *P. icarus* larvae.

### *1.5.5 Farmland Birds and Trophic Effects*

A comprehensive review by Gibbons *et al*. (2015) compiled evidence for both the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. Direct effects may be the result of ingestion of the active ingredient via the consumption of treated seed, contact toxicity from exposure to a spray application of neonicotinoids, or from the consumption of contaminated prey. The specific effects of direct consumption of treated seed on a bird species was documented in a 2017 study by Eng *et al*. (2017), using the white-crowned sparrow *Zonotrichia leucophrys*; a migratory (from southern US and Mexico to northern Canada in summer), seed-eating bird. Experimental birds were captured and fed a low (10% LD50) or high (25% LD50) dose of imidacloprid. The birds experienced 17-25% loss of body mass (low and high dose respectively), showing a rapid and substantial loss of mass within 24 hours of exposure. Their sense of direction was also affected, with birds being unable to identify the northwards direction of their migration. Negative effects were seen after consumption of the equivalent of just four imidacloprid treated oil-seed rape seeds (or less than a tenth of a corn seed) which equates to less than 1% of their daily diet. As a migratory species that uses agricultural land as a refuelling stop, they may be particularly susceptible to exposure to neonicotinoids (Eng *et al*., 2017).

Most scientific investigations have focused on those groups that are at risk from *direct* exposure to neonicotinoids, however as neonicotinoids are known to spill over into the food chain, there is a risk that they will be transported towards the higher levels of the food chain (Byholm *et al*., 2018).

The first demonstration of potential indirect effects between these pesticides and birds came from a 2014 correlational study by Hallmann *et al*. (2014). This Dutch study linked negative insectivorous bird population trends to the contamination of surface water by imidacloprid. Levels of more than 20 ppb in water tended to cause an annual population decline of up to 3.5%. This negative effect remained even after spatial effects of land use changes that are known to affect bird populations on farmland were controlled for. It highlighted that the negative impacts of neonicotinoids are experienced by species groups other than bees: if insect populations are declining, this will have a knock-on effect on insectivorous bird species. It is also possible that declines may relate to the trophic accumulation of the pesticide through the consumption of contaminated prey items (Hallmann *et al*., 2014). A recent study by Byholm *et al.* (2018) found the presence of neonicotinoid residues in blood samples of a long-distant migratory food-specialist, the European honey buzzard. The presence of neonicotinoid in the blood matched spatially with the presence of oilseed rape plant fields. This study highlights the importance of new (experimental) studies on the negative effects of neonicotinoids on species at the top of the food-chain.

Chapter 6 of this thesis describes an update to Chamberlain *et al*'s. (2000) ordination study, which tested the relationship between multivariate changes in farmland practices and bird population change between 1962 to 1995. The original study found that large scale shifts in agriculture were plausible explanations for the decline in farmland bird populations, with birds likely to be responding to a suite of interacting factors rather than individual aspects of farm management. The updated study first tests the relationship between multivariate changes in agricultural change and multi-species change in bird populations on farmland in England for the period 1996-2013. Secondly – because neonicotinoid pesticides are a new, but rapidly increasing component of arable agricultural practice in England since the mid-1990s – I also test whether there is any association between variation in bird species population trends and variation in likely exposure of different species to any direct or indirect impacts of neonicotinoid use, based on those species' ecological traits.

## 1.6 Examining the impacts of neonicotinoids on non-model organisms

Given the scale of use of neonicotinoids, their persistence in soils, their systemic nature within plants, and their capacity to leach into waterways, there is little doubt that most organisms inhabiting arable environments are exposed to them (Goulson, 2013). Therefore, the main question is whether the levels that non-target organisms are exposed to lead to significant effects at either the individual or population level. For some species groups – such as bees – there is already considerable ongoing research into this area, but gaps remain in the knowledge pool for many other organisms.

### *1.6.1. Overall aim of the thesis projects*

Collectively, the work of this thesis aimed to address some identified key knowledge gaps by establishing the effects of chronic exposures to clothianidin or thiamethoxam on ecologically important non-target organisms via field-realistic scenarios. The data are presented as the basis for further examinations on non-model organisms (those not previously selected for extensive research) specifically those species typically found in agroecosystems, which are those most likely to come into contact with neonicotinoids.Chapters 2, 3, and 5 consider laboratory-based experiments, establishing novel model systems for three different organisms across a diverse series of taxa: the hoverfly *Eristalis tenax*, the butterfly *Polyommatus icarus*, and the earth worm *Lumbricus terrestris*.

Chapter 4 considers a semi-field experiment, investigating the colonisation of contaminated microcosms by aquatic invertebrates.

Chapter 6 uses time-series data from 1996 – 2013 to assess the relationship between shifts in agricultural practices (including the degree of neonicotinoid usage) and changes in the population of multiple farmland bird species. This builds on the original analysis by Chamberlain *et al*., 2000, updating data with more recent surveys and trends.

Chapter 7 brings together findings from the preceding chapters for overall discussion, assessment of the impact of the thesis projects, and where this new information fits into policy and practise.

### *1.6.2. Choice of chemicals*

Clothianidin and thiamethoxam were chosen as focal neonicotinoids for these studies as they are two of the most commonly used neonicotinoids (Simon-Delso *et al.* 2015). Clothianidin is currently the most used seed treatment worldwide and is a breakdown product of another

commonly used neonicotinoid, thiamethoxam (Simon-Delso *et al.* 2015). Clothianidin has a reported half-life of 148-1155 days in aerobic soil, potentially exposing soil-dwelling organisms such as earthworms for extended periods of time (Jones *et al*. 2014).

This reported persistence in soil (and hence potential for long-term exposure of soil-dwelling organisms) was the justification for choosing clothianidin as a test-substance for the *L. terrestris* experiment. Further, the few studies that have considered the impact of neonicotinoids on *L. terrestris* have focussed only on the neonicotinoid imidacloprid and so there was an important gap in knowledge to try and fill. Clothianidin was also chosen for the investigation of impacts on larval development of the Common Blue butterfly *Polyommatus icarus* as we wanted to use the same neonicotinoid in the laboratory experiment that had been used in the field trials.

Thiamethoxam was used to contaminate stagnant water as it has high water solubility (average DT50 = 4,100 mg/L) which means there is high potential for it to be transported into surface water via run-off or groundwater discharge (Main *et al*. 2014). It is also a very commonly used pesticide from the neonicotinoid group (Simon-Delso *et al.* 2015) and therefore there is potential of exposure to aquatic hoverfly larvae in field realistic scenarios. Both clothianidin and thiamethoxam were used to study their effects on the colonisation of freshwater microcosms as they both persist in soil (DT50 of clothianidin is 148 – 1,155 days, and thiamethoxam's is 229 days on average (Main *et al*., 2014)), and high water solubility (thiamethoxam =4,100 mg/L; clothianidin =327 mg/L (Main *et al*., 2014)) which means there is high potential to be transported into surface waters.

In the studies reported in the following chapters, the concentrations used of each neonicotinoid were selected based on the field realistic exposures likely to be experienced by each organism; these were based on concentrations reported from sampling of UK farmland and field margins; review of surface water samples; and detected in sampled foliage.

# **Chapter 2** - Effects of chronic exposure to thiamethoxam on larvae of the hoverfly *Eristalis tenax* (Diptera, Syrphidae)

This chapter has been published as:

Basley, K., Davenport B., Vogiatzis, K., and Goulson, D. (2018). Effects of chronic exposure to thiamethoxam on larvae of the hoverfly *Eristalis tenax* (Diptera, Syrphidae). PeerJ 6:e425 doi.org/10.7717/peerj.4258

KB and DG conceived the study and were responsible for study design. KB, BD and KV were responsible for data collection. KB analysed the data, KB and DG wrote the manuscript, and BD and KV read a draft of the manuscript before submission.

## 2.1 Abstract

There is widespread concern over the use of neonicotinoid pesticides in the agro-ecosystem, due in part to their high water solubility which can lead to widespread contamination of non-target areas including standing surface water. Most studies investigating the negative fitness consequences of neonicotinoids have focused on bees, with little research on the impact on other non-target insects. Here we examined the effect of exposure on the aquatic larval stages of the hoverfly *Eristalis tenax* L. (Diptera: Syrphidae) to a range of concentrations (control, 5ppb, 15ppb, 50ppb, 100ppb and 500ppb) of the neonicotinoid thiamethoxam; no published studies have thus far examined the effects of neonicotinoids on hoverflies. Survival was significantly lower when exposed to 500ppb thiamethoxam, but this concentration exceeds that likely to be found in the field. We observed no effect on survival, development, or any latent effects on adult activity budgets resulting from exposure to lower concentrations (up to 100ppb). Our results suggest that *E. tenax* exposed as larvae to thiamethoxam are unlikely to be negatively impacted by this neonicotinoid under field conditions.

## 2.2 Introduction

Beneficial insects play an essential role in the functioning of natural ecosystems and pollination is perhaps the best documented of the ecosystem services provided by insects (Vanbergen & Insect Pollinators Initiative 2013). The economic value provided by wild pollinators is on par with that provided by managed honeybees (Kleijn *et al*. 2015), it is therefore vital to understand the causes behind the reported widespread population declines of many pollinators (Biesmeijer *et al*. 2006; Carvalheiro *et al*. 2013; Potts *et al*. 2010; Burkle *et al*. 2013; Jauker *et al*. 2012).

In many countries, land use is dominated by agriculture and this has been subject to major change due to the industrialisation of food production and the advent of increased mechanisation and chemical-input (Robinson & Sutherland 2002). Neonicotinoid pesticides, first introduced to the global market in the mid-1990's (Jeschke *et al*. 2011), have been rapidly adopted and are now used in over 120 different countries, on hundreds of different crops, via soil drenches, sprays and most commonly, as seed dressings (Morrissey *et al*. 2015). When applied as a seed treatment, 1-2% of the active ingredient is released onto the wind as dust (Tapparo *et al*. 2012). Some of the active ingredient from the seed dressing is subsequently taken up by the plant, however owing to neonicotinoid's high water solubility, on average

about 90% of the total active ingredient applied is lost to the soil (Goulson, 2013). This can lead to widespread contamination of farms and the surrounding environment, with potential for impact on both pollinators and predatory insects (Botías *et al*. 2016; Botias *et al*. 2015; Krupke *et al*. 2012; Rundlöf *et al*. 2015; Jones *et al*. 2014).

Surface waters, including puddled water, ditches, irrigation channels and streams in and near farmland have been found to be contaminated by neonicotinoids (Morrissey *et al*. 2015; Van Dijk *et al*. 2013; Samson-Robert *et al*. 2014, Main *et al*. 2014, Schaafsma *et al*. 2015). For example, thiamethoxam, one of the most commonly used pesticides from the neonicotinoid group (Simon-Delso *et al*. 2015), has a relatively long half-life in soil and high water solubility (average DT50 = 229 days, 4,100 mg/L) which means it is persistent in the environment with high potential to be transported into surface water via run-off or groundwater discharge (Main *et al*. 2014). A recent survey of water monitoring literature focussing on surface water contamination by neonicotinoids, found thiamethoxam levels to range from 0.001ppb to 225ppb (Morrissey *et al*. 2015). Even low levels of neonicotinoids have been associated with negative effects on aquatic invertebrates, evident at both the individual and population level (Pisa *et al*. 2015); for example the $LC_{50}$ for imidacloprid and the mayfly *Ceriodaphnia dubia* is 2.1ppb (Chen *et al*. 2010).

Neonicotinoid pesticides act as agonists of the nicotinic acetycholine receptors, resulting in excitation, paralysis and death of the target insect (Moens *et al*. 2011). Numerous studies have raised concerns over the use of neonicotinoid pesticides and the risks to bees, suggesting that exposure to field-relevant doses can impair pollen collection, increase worker mortality, reduce the production of new queens, weaken the bee's immune system and affect the weight of honeybee queens (Gill & Raine 2014; Gill *et al*. 2012; Whitehorn *et al*. 2012; Di Prisco *et al*. 2013; Gajger *et al*. 2017). However, little research has focused on other non-target insects.

Hoverflies (Syrphidae) are often considered to be the second most important pollinators after bees (Larson *et al*. 2001). Evidence suggests *Eristalis tenax* (Linnaeus) has pollination value in open and closed crop production systems, and at high densities has a pollination effort comparable to the efficacy of small honey bee colonies (Jauker *et al*. 2012). Some species of hoverfly are also valued biocontrol agents since their larvae eat aphids (Ramsden *et al*. 2016). Additionally, approximately half of all hoverflies have saprophagous larvae (Gilbert *et al.,* 1994), these species play an essential part in the decomposition and recycling process of a wide variety of materials, including compost, dung and dead wood, by breaking up and

aerating the substrate as they move through it (Gilbert 1985). Therefore, it is prudent to encourage hoverfly populations on farmland to maintain a healthy functioning ecosystem, at a time where other pollinators like bees are suffering serious declines due to a wide range of stressors (Goulson *et al*. 2015). In addition, we need to ascertain if there are any latent sublethal effects on adult function stemming from larval exposure which may impair their value as pollinators.

The repeated application of insecticides can lead to a significant loss of dipteran larvae and a potential accumulation of dead organic material in surface water (Sanchez-Bayo 2011); however, there is a dearth of studies investigating the impact of neonicotinoids on the aquatic larvae of Diptera (Pisa *et al*. 2015). The authors are aware of no published studies that have investigated the impact of neonicotinoids on Syrphidae and, due to the inherent differences in physiology among species, considerably more research is required (Pisa *et al*. 2015). Here, we experimentally test the effect of field-realistic doses of a commonly used and highly persistent neonicotinoid, thiamethoxam, on the development of the aquatic larvae, and latent effects in adult behaviour, of the hoverfly *E. tenax.*

## 2.3 Methods

*Study organism and rearing method*

Female *E. tenax* deposit eggs on the surface of stagnant water or decaying material and, under laboratory conditions, eggs hatch within 2-3 days (K. Basley 2016, personal observations). The aquatic larvae filter feed on microbes in decaying organic matter, and respire using an extended anal segment used as a breathing tube (Rotheray 1993). Once fully grown, larvae exit the aquatic habitat in search of a dry shaded place in which to pupate. Adults feed on both pollen and nectar and, in the UK, can be found on the wing from late March to early December (Ball & Morris, 2013).

To produce a suitable silage substrate for oviposition, two weeks before the beginning of the experiment, three 14L buckets were filled with a mixture of grass clippings and water. Fresh grass clippings were obtained from the University of Sussex campus where there is no recorded history of neonicotinoid usage. Three more buckets were filled with a larch (*Larix decidua)* sawdust and water mix. Buckets were covered in a very fine insect proof muslin, to prevent any insects from ovipositing in the mixture. All six buckets were left outside to allow to decompose for two weeks. The grass clippings were then strained through muslin to produce

'grass silage', and the collected water, designated 'silage water,' was retained. The sawdust buckets were also strained, the sawdust solids were retained but the water was discarded. The grass silage and sawdust solids were further squeezed to remove excess water and used in varying ratios to produce an oviposition tray substrate or to create either a holding lagoon or neonicotinoid-treated experimental lagoon substrate.

To obtain larvae of a known age, prospecting female *E. tenax* were collected from a large heap of grass clippings on the University of Sussex campus (50° 52' N, 0° 4' W) between May and August 2016, one week before the start of each experimental round. Females were returned to the laboratory and placed inside mesh cages (60cm x 45cm x 60cm) under fluorescent light and provided with untreated pollen (Biobest via Agralan Ltd), 15% sucrose solution w/v, and mineral water (ASDA Stores Ltd, own brand). A tray (30cm x 40cm x 6cm) filled with a 2:3 mixture (by weight) of grass silage and 'silage water' (see above for preparation) with dried leaves and twigs placed on the surface (henceforth referred to as "oviposition trays") was placed in each cage.

Once females were introduced to the cages, oviposition trays were checked twice daily for eggs and once eggs had been laid they were removed to a smaller 0.2L plastic cup, filled with 60g of a grass silage : silage water (2:3 mix), and twigs. Once hatched, larvae remained in these 'holding lagoons' before being transferred to the neonicotinoid-treated experimental lagoons at 5 days of age as this was the time when they were large enough to handle (a body length no smaller than 5mm).

*Pesticide exposure*

Neonicotinoid-treated experimental lagoons were created by thoroughly mixing together sawdust solids and grass silage in a 4:1, ratio (hereafter referred to as 'substrate'). Sixty grams of the substrate was then added to 0.2L plastic cups (hereafter referred to as 'lagoons'; E Rotheray, 2015, personal communication) and each placed in a tie-top plastic freezer bag surrounded by dried leaves which had been sieved to remove smaller pieces of detritus (Figure 2.1).

In order to contaminate the larval growth substrate, a mixture of silage water (700ml) and bottled water (1L) (ASDA, own brand) was contaminated to six different levels with analytical grade thiamethoxam using stock solutions (Sigma-Aldrich, Gillingham, UK): 0ppb (control), 5, 15, 50, 100 and 500 ppb as a positive. One hundred and fifty millilitres of each treatment

solution was added to each treatment lagoon and stirred thoroughly with a small stick which was left in the lagoon. Five day old larvae (from date of hatching), were removed from the holding lagoons, gently rinsed in bottled water, blotted dry with paper towel and weighed with a 0.001g resolution balance (Precisa 125A, Newport Pagnell, Buckinghamshire, UK) before being placed into the treatment lagoons.

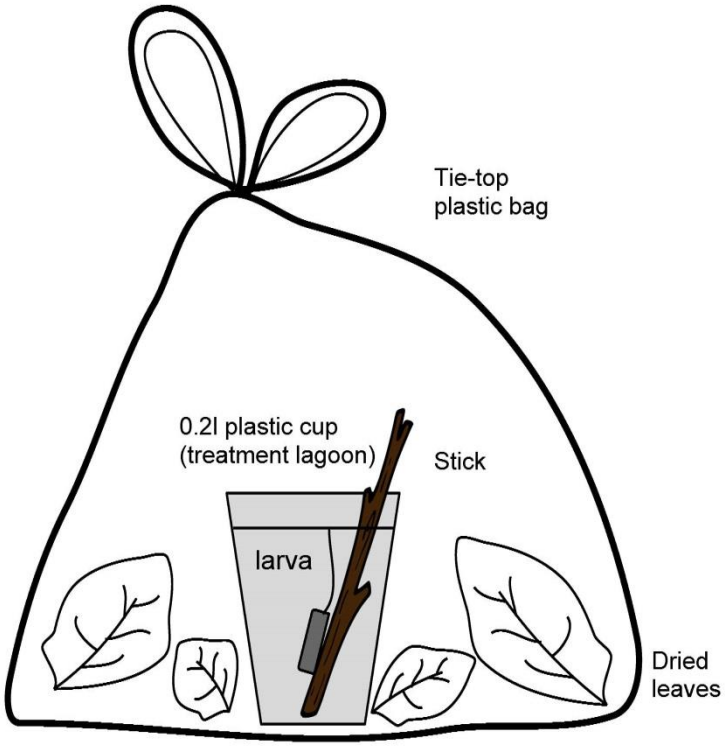



**Figure 2.1** Treatment lagoons were placed inside a large tie-top plastic bag and surrounded by sieved dried leaves (to be used as a pupation substrate). The stick allowed larvae to crawl out of the treatment lagoon and pupate.

Larvae were randomly assigned to treatment groups with ten individual replicates per treatment group (60 larvae in total per full experiment). Larvae were exposed to thiamethoxam from the day they were introduced to the treatment lagoon, to the day they started to pupate. The full experiment was repeated four times (240 larvae), and each separate experiment was populated with eggs from a different female, to ensure that any genetic variation in tolerance to thiamethoxam did not confound the experimental design (Hemingway *et al*. 2004). Lagoons contained sticks to allow larvae to climb out to pupate, but were covered with a plastic bag to prevent larvae from escaping. The dried leaves acted as a pupation site. Throughout the experiment, lagoons were kept in a dark room (21$^{o}$C) to prevent light degradation of thiamethoxam helping to ensure that there was an equal distribution of thiamethoxam through the lagoon profile (Peña *et al*. 2011). Ottenheim and Holloway (1994)

investigated the effect of light and diet on the laboratory development of *E. arbustorum* and found no detrimental effects of light regime on survival or developmental phase of larvae, although immature development was slightly faster under bright conditions.

*Larval Development*

Following Rotheray *et al*. (2016), larval growth was monitored by increase in mass. Every three days the larvae were removed from the treatment lagoons, gently rinsed in mineral water (ASDA, own brand) and blotted dry before being weighed and replaced in the lagoon. If the larvae could not be located, the bag of leaves was searched for larvae or pupae. To ensure there was no degradation of the thiamethoxam, all measurements took place under red light. If a larva was found that had exited the lagoon prematurely and was not pupating, the replicate was removed from the experiment. Pupal mass and date of pupation (+/- 3 days) were also recorded. Once pupation had commenced, remaining non-pupating replicates were checked for pupation twice daily. Pupae were weighed on a 0.001g resolution balance, and individually placed in labelled 50 mL tubes with netting secured over the opening, with a small amount of tissue paper to absorb any excess moisture. These tubes were stored in the dark at 21$^{o}$C and five days after pupation were checked twice daily for emergence.

*Adult measurements*

Upon emergence, adults were colour-marked on their thorax denoting their treatment group with a spot of non-toxic enamel paint, released into a flight cage (60cm x 45cm x 60cm), and provided with pollen, water, and a 15% sucrose solution for one week. To observe and compare the behaviour of individual flies, 7-day old adults were individually placed into a smaller cage of the same design (30cm x 20cm x 25cm), provided with water and 15% sucrose solution in feeders and a small amount of pollen. They were given one minute to acclimatise. Using an instantaneous sampling technique (following similar protocols in Gilbert (1985)), behaviour of all adults was then recorded for 10 minutes. These behavioural activity budgets were categorised as: stationary, grooming, walking, flying, probing through the cage netting with their proboscis, feeding on nectar, pollen or water (grouped together as feeding) and moving which involved remaining stationary whilst making small jerking motions of their body.

*Statistical Analysis*

All statistical analyses were carried out using SPSS (v. 21 IBM SPSS Inc., Armonk, NY, USA). Data from the four experiment replicates were pooled for all analyses. The significance threshold was set at 0.05. Although ideally statistical power calculations would have been done prior to

the experiment, the effect size and likely data variability were unknown due to the novel nature of this protocol. The number of replicates were therefore maximised within the time constraints of the experimental husbandry.

*Larval development*

Data were tested for normality using the Shapiro-Wilk statistic and visual inspection of Q-Q plots, and homogeneity of variance was tested using Levene's statistic. A one-way ANOVA was used to determine the effect of thiamethoxam on pupal weight. Due to deviations from normality a Kruskall-Wallis H test was used to investigate the effect of treatment on larval development time (5-day old larvae to pupation). Larval weight data were log-transformed to achieve normal error distribution and was used as our response variable. These were compared between treatment groups using a generalised linear mixed model (GLMM, link function, normal distribution) with treatment (thiamethoxam presence or control) and time (day 3, 6, 9, or 12) as fixed factors, 'experiment round' (1, 2, 3 or 4) was included as the random effect, and 'scaled identity' for the repeated measures covariance structure (where a structure has constant variance and there is assumed to be no correlation between any elements). We first fitted a full model and systematically omitted interaction terms if they did not increase model fit. Model fit was compared using the Akaike Information Criterion (AIC). AIC was also used in selecting the repeated covariance type in models with repeated measures structure. Fisher's exact test (2 x 6) was used to analyse the distribution of count data between treatment type and the likelihood to exit a lagoon prematurely or remain in lagoon.

*Survival Analysis*

Larvae that reached the pupal stage were counted as survivors, irrespective of whether they later successfully completed metamorphosis (Haider *et al*. 2013). Survival of the larvae across the treatment groups was analysed using Kaplan-Meier survival analysis, and the log-rank test with a Bonferroni correction was applied to test for differences between survival distributions across treatment groups. Replicates where larvae were found in the leaves but were not pupating were completely removed from the experiment. Once individuals reached pupation they were treated as 'censored' data (irrespective of what happened to them after this point since this no longer considered 'larval survival'). Since the censored data between treatment groups were different (specifically between the 500ppb group and the other groups), the percentages were also reported, as is standard for Kaplan-Meier analysis (Table 2). Median lethal concentration ($LC_{50}$) was calculated by probit regression analysis.

**Table 2.** Larval survival, development time and average pupal weight from six different larval populations reared in substrate contaminated with thiamethoxam. Note; treatments sharing the letter did not differ significantly at $P< 0.05$ (post-hoc test; pairwise log-rank).

| Treatment Group | Number of larvae that reach pupation (total n of group) | Survival (%) | Average Pupal weight (g) ± SD |
|---|---|---|---|
| Control (A) | 30 (36) | 83.3 | 0.249 ± 0.0049 |
| 5ppb (A) | 27 (36) | 75 | 0.240 ± 0.0056 |
| 15ppb (A) | 20 (33) | 63.6 | 0.255 ± 0.0086 |
| 50ppb (A) | 27 (35) | 77.1 | 0.250 ± 0.0064 |
| 100ppb (A) | 27 (35) | 77.1 | 0.247 ± 0.0057 |
| 500ppb (B) | 5 (38) | 13.2 | 0.227 ± 0.0129 |

*Adult Behaviour*

The total amount of time spent carrying out each behaviour was compared between treatment groups. Assumptions of normality were not met for each group of the independent variables as defined by the Shapiro-Wilk statistic and visual inspection of histograms, and so individual non-parametric Kruskal-Wallis H-tests were used to investigate the effect of thiamethoxam treatment on adult behaviour. The animals had N states in the time budget which meant that the variables were unavoidably linked by summing to 100%. However there were no significant effects of treatment on adult behaviour patterns, so this did not create further problems in the analysis.

## 2.4 Results

*Larval development*

Across treatments, 27 larvae exited the lagoons prematurely and were found in the dried leaves. By the end of the experiment, for the control, 5ppb and 50ppb groups, four larvae (of 40 replicates in that treatment group) had exited prematurely (10%). Most larvae that were found in the leaves were in the 15ppb group (7/40, 17.5%) with the least in 500ppb (2/40, 5%); but overall there was no effect of treatment on exiting larvae (Fisher's Exact Test, p= 0.656). The lower figure for the positive control (500ppb) is probably due to the elevated mortality levels of larvae in this treatment. These replicates were removed from all further statistical analyses.

There was no significant effect of treatment on development time, which was 9-13 days (Kruskall-Wallis; *H*(5)=3.367, p= 0.644; median for all groups – 12 days), and no effect of treatment on pupal weight (One-way ANOVA, $F_{5, 129}$=1.029, p=0.403). Larval weight did not significantly differ between treatment groups (GLMM; $F_{5, 762}$=0.326, p= 0.897).

*Survival*

Mortality across the six treatment groups was significantly different (Kaplan-Meier, log rank; $\chi^2(5)$=122.27, p = <0.001) and post-hoc pairwise comparisons showed significant differences between all treatment groups and the 500ppb group (Kaplan-Meier analysis, pairwise log-rank test: control-500ppb $\chi^2(1)$=50.172, p=<0.001; 5ppb-500ppb, $\chi^2(1)$=39.272, p=<0.001; 15ppb-500ppb, $\chi^2(1)$=35.431, p=<0.001; 50ppb-500ppb, $\chi^2(1)$=36.280, p=<0.001; 100ppb-500ppb, $\chi^2(1)$=41.112, p=<0.001) (Figure 2.2). Percentage survival was lowest in the 500ppb group (13.2%), and highest in the control (83.3%) (Table 2). The $LC_{50}$ for thiamethoxam and *E. tenax* was 215ppb (95% CI [113.5, 447.3]).

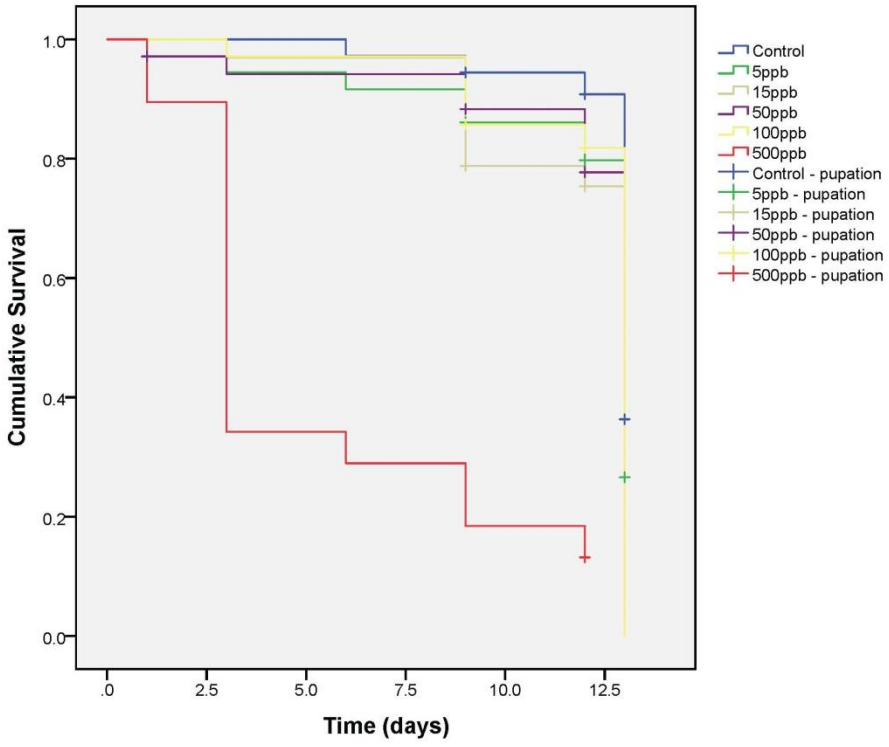



**Figure 2.2** Cumulative survival of *Eristalis tenax* larvae (*N* = 33-38 per treatment) when reared in substrate contaminated with five different concentrations of thiamethoxam, plus control. Crosses indicate individuals that reached pupation (censored data). Many individuals pupated at the same time and so crosses are nested underneath one another. Post-hoc pairwise comparisons (Kaplan-Meier analysis, pairwise log-rank tests) showed significant differences between all groups with 500 ppb.

*Adult behaviour*

Distribution shapes were similar for all behaviour groups across treatments as assessed by visual inspection of a box plot (Appendix 2A). Median scores for all behaviours were not significantly different across treatments (Kruskall-Wallis; time spent: stationary $H(5)=4.989$, $p=0.417$; grooming $H(5)=8.217$, $p=0.145$; walking $H(5)=6.960$, $p=0.224$; flying $H(5)=0.980$, $p=0.964$; probing $H(5)=3.188$, $p=0.671$; feeding $H(5)=7.497$, $p=0.186$; moving $H(5)=5.571$, $p=0.350$).

## 2.5 Discussion

While thiamethoxam has been detected in waterbodies on and near to farmland (Samson-Robert *et al*. 2014) with the potential for harming non-target species (Pisa *et al*., 2015; Morrissey *et al*. 2015) we report little or no effect of larval exposure to field relevant doses of the neonicotinoid thiamethoxam via contaminated substrate. Our results indicate that *E. tenax* larvae are insensitive to field realistic doses of thiamethoxam with no significant likelihood of direct mortality, or impacts on growth, development time or activity budgets in the resulting adults. These are the first known published data on the effects of a neonicotinoid on the insect family Syrphidae.

Within the field of aquatic toxicology, the chironomids (Diptera) are widely used in laboratory tests, with most work being undertaken at the organismal level by measuring larval survival and growth (Saraiva *et al*. 2017). A comprehensive review by Morrissey *et al.* (2015) looked at the lethal concentration in water ($LC_{50}$) and the $EC_{50}$ values (where 50% of the pesticide's maximal effect is observed) for 214 acute (24-48h) and chronic studies (7-28 days) for 48 species of aquatic invertebrate species. The geometric mean taken from the range of the $LC[E]_{50}$s for all Diptera and neonicotinoids tested was 32.9 ppb, and was 9.3ppb for *Chironomous dilutes* (Diptera: Chironomidae) specifically. Aquatic invertebrate species also appear to vary in their sensitivity with *C. dilutes* being found to be the most sensitive of the three most common aquatic invertebrate species tested (compared to *Daphnia magna* (Cladocera; geometric mean: 23,690ppb)) and *Gammarus pulex* (Amphipoda; geometric mean: 235.8ppb)) (Morrissey *et al.* 2015), which emphasises the importance of testing a wide range of species in addition to a range of chemicals.

From this same review, only two studies examining effects of thiamethoxam on Diptera (Culicidae) were reported: *Aedes aegypti* (24h) and *Chironomus riparius* (48h) resulting in an $LC[E]_{50}$ of 183 and 35 ppb respectively. Thiamethoxam is an order of magnitude less toxic than

two other neonicotinoids, imidacloprid and clothianidin, to all life stages of *C. dilutes* over a 14-day exposure. The 14-day median lethal concentrations for imidacloprid, clothianidin and thiamethoxam were 1520ppb, 2410ppb and 23600ppb. The 40-day median effect concentrations (emergence) for imidacloprid, clothianidin and thiamethoxam were, 390ppb, 280ppb, and 4130ppb, respectively (Cavallaro *et al*. 2016). The focus of this study was on thiamethoxam, as the most widely used seed dressing on oilseed rape in the UK. While clothianidin displays a lower 40-day median effect in *C. dilutes* than thiamethoxam, other studies have demonstrated that toxicity can differ strongly between closely related species – the chronic $LC_{50}$ of imidacloprid to *Chironomus tentans* is just 0.91ppb (Stoughton *et al*. 2008), for example, so comparisons between the susceptibilities of different Diptera to different neonicotinoids are difficult to draw confidently. A lack of studies on the effects of thiamethoxam on Diptera prevents much in the way of comparison. Our study estimated the thiamethoxam $LC_{50}$ for *E. tenax* to be much higher than 215ppb. It seems possible that thiamethoxam has a generally lower toxicity to aquatic invertebrates when compared to imidacloprid or clothianidin, but clearly more comparative studies are needed to draw firm conclusions.

Earlier larval instars have been consistently shown to be more sensitive to contaminants due to differences in biomass and bioaccumulation after exposure to a contaminant (Heinis *et al*. 1990). Our experiment commenced with five day old larvae (which was essential to allow handling of larvae), so it is possible that if eggs were laid directly in contaminated water, hatching or commencement of growth could be more adversely affected.

Despite ensuring the lagoons were not exposed to UV light for the duration of the experiment (as UV is the major component contributing to thiamethoxam's photolytic decomposition (Gupta *et al*. 2008), it is possible that during the experiment the thiamethoxam degraded over time due to the physicochemical properties of the matrix or bacterial action. Thiamethoxam in contaminated waste water rapidly degrades in darkness and this degradation has been attributed to the presence of microorganisms using the neonicotinoid as an energy source; a lagged effect was noticed as the microorganisms adapted to using the thiamethoxam (Peña *et al*. 2011). It is thus possible that the bacterial content of the lagoons resulted in biodegradation of the pesticide. However, if so, we would expect much the same to occur in the field, unless the contamination were being topped up from new sources.

Larvae of *E. tenax* mature in stagnant, anaerobic ponds and water-courses where they filter-feed on microbes associated with rotting organic material and faecal matter (Hayes *et al*. 2016). It is possible that, due to being adapted to exploit these fetid environments, they are naturally robust and capable of coping with toxins. It is also feasible that their cuticle is impermeable therefore may prevent absorption of the chemical, reducing contact toxicity.

Interestingly, some larvae (11%) prematurely exited the lagoon before pupation, with some exiting just three days after transfer. We found no effect of treatment on the likelihood to exit a lagoon. We therefore hypothesise that larvae may be capable of detecting different conditions which may be unfavourable compared to those in which they started development. Larvae are known to travel up to 10 metres in search of favourable pupation habitats (Fischer *et al*. 2006), so exiting to search for more favourable larval habitats, or the original habitat from which they were displaced, may be a reason for this observation. Despite this, we believe that the experimental set-up was still fit-for-purpose. Further, it is possible that as the larvae were kept in the dark for the duration of the experiment (with an absence of a typical day:night light regime) this could have impacted their behaviour.

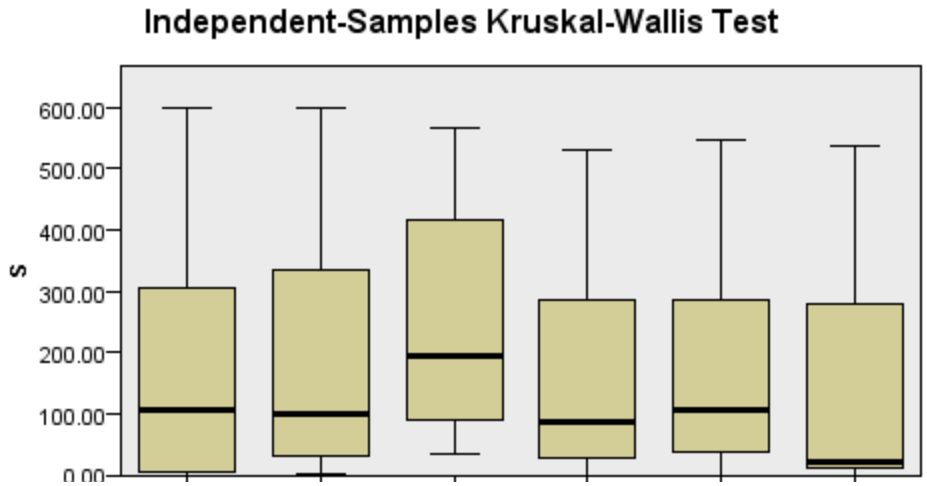
Evidence from studies on honeybees and bumblebees suggest that there is a latent effect of larval neonicotinoid exposure on the behaviour of the resulting adult. For example, larvae of *Apis cerana* (Apidae) exposed to low doses of imidacloprid (0.24ng/bee) exhibited significantly impaired olfactory learning when tested as adults (Tan *et al*. 2015); the same effect was seen in *Apis mellifera* alongside higher brood mortality and reduced adult lifespan (Peng & Yang 2016). Exposure to thiamethoxam during development of the bumblebee can result in decreased memory function (Stanley *et al*. 2015), and reduced emerging queen body weights, reduced ovary weights, and lowered sperm counts in the honeybee (Gajger *et al*. 2017). In this study, we found larval exposure to thiamethoxam and its metabolites to have no latent effect on in-situ adult hoverfly activity budgets, though we did not test for effects of high level behaviours such as learning and memory. It is noted that the nervous system of adult insects is very different from that of the larvae, with the structures targeted by neonicotinoids, such as the mushroom-bodies in the brain, being undeveloped in the larvae (Farris *et al*. 1999). Further work is warranted on adult exposure to pollen and nectar containing field-relevant levels of neonicotinoids, as they pose the same potential risk of harm to hoverflies as they do to bees.

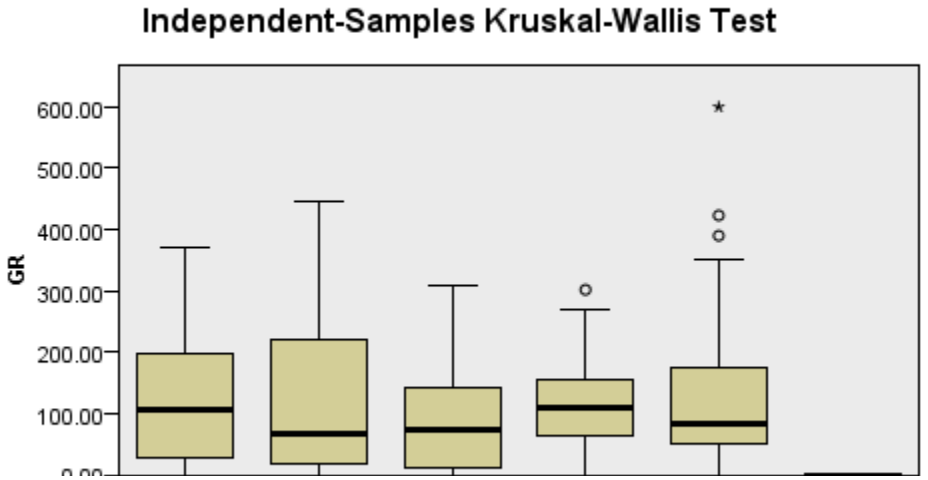
Research is most often focused on the effects of singular chemical exposures However, fields can be treated with a large number of chemical compounds, with pesticides regularly applied

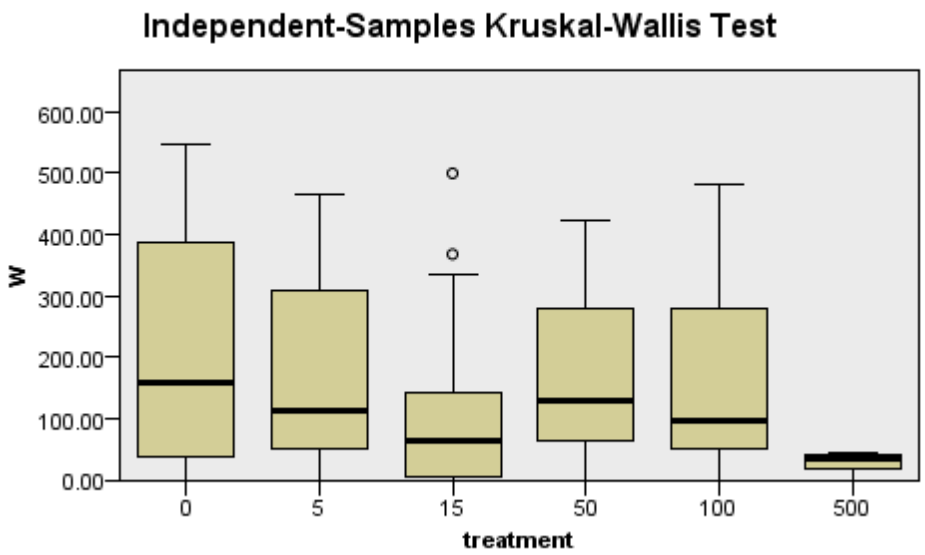
as mixtures of similar or different active ingredients being common practice (Cavallaro *et al*. 2016; Botías *et al*. 2017). This potential exposure to a cocktail of chemicals in agricultural run-off is not addressed in this study and has not been commonly addressed in the wider field of investigations on the effect of pesticides on non-target organisms in general. Further research should examine exposure to field realistic mixtures of chemicals (Rodney *et al*. 2013).

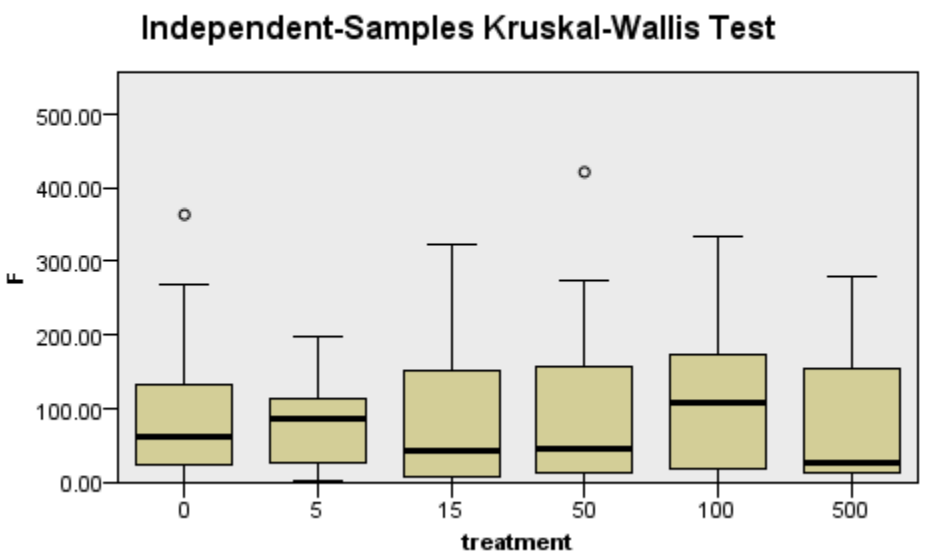
In summary, we found that thiamethoxam exposure results in elevated mortality of *E. tenax* larvae only at concentrations above those normally found in field-realistic situations. The larvae of this species appears to be less sensitive to thiamethoxam than some other aquatic insects that have previously been examined. Further research is required to investigate possible adverse effects via adult exposure, or from larval exposure to other neonicotinoids and currently-used complex mixtures of pesticides. Farmland management may benefit from including hoverfly larval habitat to maintain an important pollinating species which, at least in the larval stage, appears to not be highly susceptible to at least one commonly used pesticide.

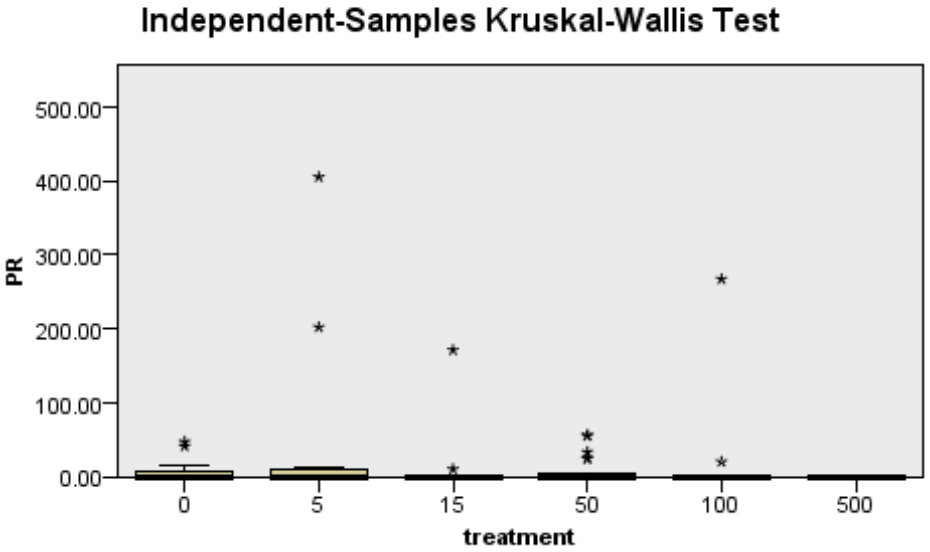
**Appendix 2A – Boxplots showing distribution shapes of adult *E. tenax* behaviour.** (Behaviours are as follows: S = stationary, GR= grooming, W= walking, F= flying, PR= probing, N= feeding, M= moving).

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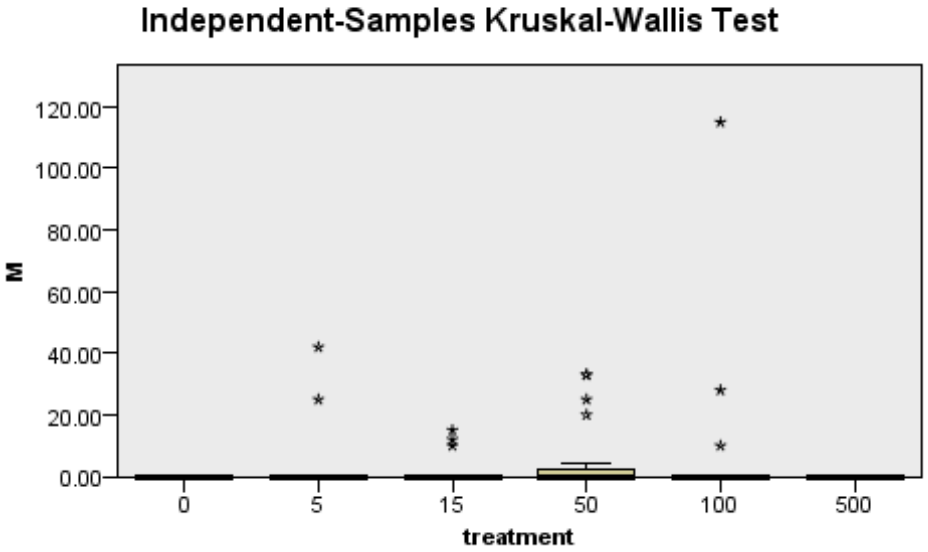
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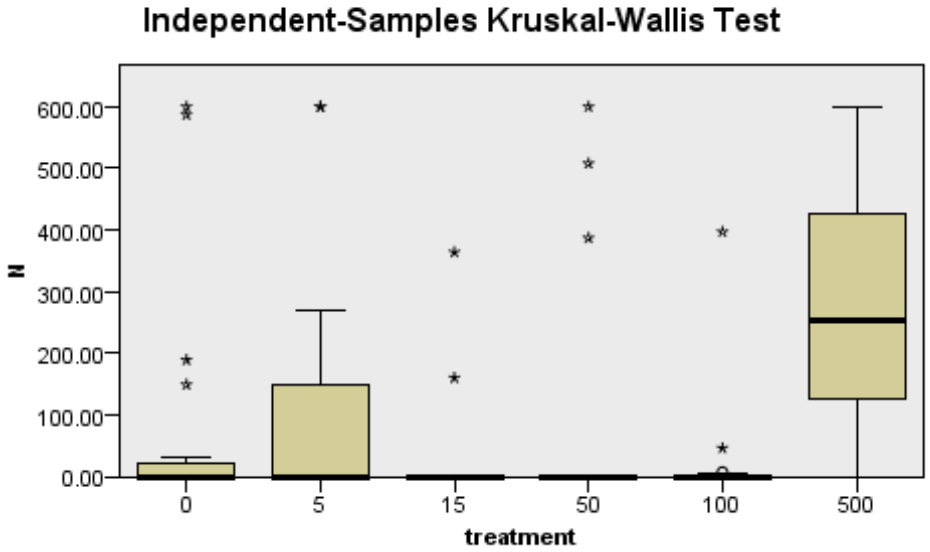
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# **Chapter 3** - Effects of chronic exposure to clothianidin on the earthworm *Lumbricus terrestris*

This chapter has been published as:

Basley, K., and Goulson, D. (2017). Effects of chronic exposure to clothianidin on the earthworm *Lumbricus terrestris*. PeerJ 5:e3177; DOI 10.7717/peerj.3177

KB and DG conceived the study. KB was responsible for all aspects of data collection and for study design. KB and DG were collectively responsible for data analysis and writing of the manuscript.

## 3.1 Abstract

Although neonicotinoids are targeted at insects, their predominant use as a seed dressing and their long persistence in soils mean that non-target soil organisms such as earthworms are likely to be chronically exposed to them. Chronic exposure may pose risks that are not evaluated in most toxicity tests. We experimentally tested the effect of field-realistic concentrations of a commonly used neonicotinoid, clothianidin, on mortality, weight gain, and food consumption to assess the impacts of chronic exposure over four months on fitness of *L. terrestris* individuals. We undertook three separate experiments, each with different exposure routes: treated soil only (experiment A), treated food and soil combined (experiment B) and treated food only (experiment C). Mortality was negatively affected by exposure from treated soil only with greatest mortality observed in the groups exposed to the two highest concentrations (20ppb and 100ppb), but no clear effect on mortality was found in the other two experiments. When clothianidin was present in the food, an anti-feedant effect was present in months one and two which subsequently disappeared; if this occurs in the field, it could result in reduced rates of decomposition of treated crop foliage. We found no significant effects of any treatment on worm body mass. We cannot rule out stronger adverse effects if worms come into close proximity to treated seeds, or if other aspects of fitness were examined. Overall our data suggest that field-realistic exposure to clothianidin has a significant but temporary effect on food consumption and can have weak but significant impacts on mortality of *L. terrestris.*

## 3.2 Introduction

Neonicotinoids are the most widely used group of pesticides in the world (Jeschke *et al*. 2011). Their leaching potential and systemic properties mean that many non-target organisms in agricultural landscapes are likely to be exposed (Goulson, 2013), and their current prophylactic use on many arable crops presents a potential for large scale contamination of non-target areas. Neonicotinoids are often applied as seed dressings (Jones *et al*. 2014), with typically 94% of the active ingredient applied to the crop seed entering the soil rather than the crop (Goulson, 2013). Residues of these compounds have been detected in soil more than three years after introduction via seed treatments (Botías *et al*. 2016). Clothianidin, a commonly used neonicotinoid, has a reported half-life of 148-1155 days in aerobic soil, potentially exposing soil-dwelling organisms such as earthworms for extended periods of time (Jones *et al*. 2014). It is this reported persistence that is

amplifying the concern surrounding the impact of neonicotinoids on non-target organisms.

The application of agricultural products such as neonicotinoids for the protection of agricultural and horticultural crops has been shown to introduce these compounds to the drilosphere (the part of the soil which is influenced by earthworm secretions and castings), where the soil acts as a sink for agricultural products (Givaudan *et al*. 2014). Neonicotinoids can also compromise the function of soil organisms that contribute to soil fertility which may limit crop yield (Moffat *et al*. 2016). Their presence in the soil profile poses a hazard to resident worm populations, as the same neural pathways that are the target of neonicotinoids in pest species, are also present in earthworms (Volkov *et al*. 2007). Acting as Nicotinic Acetycholine Receptor agonists, very low levels can significantly disrupt neural functioning in bees (Piiroinen *et al.,* 2016), so if the negative effects of the neonicotinoid are similar to those for other non-target insects (Pisa *et al.,* 2015), the worm's critically important role in the maintenance of soil properties could potentially be affected. Exposure can either be by direct physical contact with a treated seed or contaminated soil or soil water. Moreover, it is typical for earthworm species to ingest soil particles as they burrow, hence presenting an oral route of exposure to the compounds (Pisa *et al*. 2015).

The majority of studies investigating the impact of neonicotinoids on earthworms have focused on *Eisenia fetida*, with the range of reported lethal concentrations based on this species and little consideration given to the sensitivities of other species (Pisa *et al*. 2015). *E. fetida* are compost worms, and so are not typically found in areas where neonicotinoids are in use, preferring warm and moist habitats with a ready supply of fresh compost material. They are also claimed to be less sensitive to environmental toxicants than other earthworm species (Dittbrenner *et al*. 2010), and so results from these test species provide little insight into the potential impact of pesticides on earthworms in arable ecosystems. A recent review exploring the biochemical and molecular markers as indicators of the accumulation of pollutants, specifically pesticides, reported varying levels of biomolecules in different parts of the earthworms which indicated varying sensitivity of earthworms to different xenobiotics (Tiwari *et al.,* 2016).

*L. terrestris* is commonly found in grasslands and lawns, especially when the ground is left undisturbed (Sherlock 2012) and is more representative of species found on agricultural land and in field margin soils than *E. fetida* (Nuutinen *et al*. 2011); its widespread geographical

range and frequently high abundance make it a special target for concern and study, since it is likely to play a major role in contributing to soil health (Tomlin, 1992).

Anecic worm species such as *L. terrestris* live and feed on the soil and are of particular benefit to arable soils where worms can constitute up to 80% of total soil animal biomass (Pisa *et al*. 2015). Their wide and deep-penetrating burrows open up the structure of compacted and clayey soils by enabling water infiltration (Nuutinen *et al*. 2011), and soil fertility is enhanced by the breakdown of plant litter and the mixing of this litter with the soil (Pisa *et al*. 2015). Physical disturbance of the soil by tillage and ploughing can have strong negative effects on the abundance of *L. terrestris* and so higher population densities are often found in field margins which may act as source areas for the worm, supporting population growth within the field (Nuutinen *et al*. 2011). As these worms feed at the soil surface they are likely to be exposed to higher concentrations of pesticides as agrochemical concentration is often higher at the soil surface (Chagnon *et al*. 2015).

To date, the few studies that have considered the impact of neonicotinoids on *L. terrestris* have focussed only on the neonicotinoid imidacloprid (Table 3). Studies showed little or no measurable impact on survival at 4ppm (Dittbrenner *et al*. 2012), but with a moderate decrease in body mass and large decrease in cast production being observed at 0.66ppm (Dittbrenner *et al*. 2010). Cast production was negatively affected at 0.66ppm and 0.189ppm (Capowiez *et al*. 2010).

**Table 3.** Impact of neonicotinoid imidacloprid on L. terrestris. Lowest effective concentration is the lowest concentration at which a significant effect was reported. 0 = little or no measurable effect, - = moderate decrease, -- = large decrease. Table adapted from Pisa *et al*. (2015).

| Measured endpoint | Impact | Lowest effective concentration | Duration of exposure to contaminant | Study |
|---|---|---|---|---|
| Survival<br>Avoidance | 0<br>0 | 4 ppm | 14 days | (Dittbrenner *et al*. 2012) |
| Burrowing | - | 2 ppm | 7 days | (Dittbrenner *et al*. 2011) |
| Feeding activity<br>Abundance | -<br>- | 43 mg $m^{-2}$ | 6 weeks | (Tu *et al*. 2011) |
| Body mass change<br>Cast production | -<br>- | 0.66 ppm<br>0.66 ppm | 7 days<br>7 days | (Dittbrenner *et al*. 2010) |
| Cast production<br>Body mass change | -<br>- | 1.89 ppm<br>0.189 ppm | 7 days<br>7 days | (Capowiez *et al*. 2010) |

The authors are aware of no published studies that have investigated the impact of chronic exposure of clothianidin on *L. terrestris.* Clothianidin has recently become the most commonly used neonicotinoid in the UK (DEFRA 2014), and is regularly used for seed, foliar, and soil treatments (Jeschke *et al*. 2011). One study of agrochemical toxicity to *E. fetida* found clothianidin to be the most toxic of 45 pesticides tested*,* with an LC50 value of 0.28 µg $cm^{-2}$ from a filter paper contact test. When tested in artificial soil for 14 days, clothianidin showed the highest intrinsic toxicity against *E. fetida* with an LC50 values of 6.06 (5.60–6.77) mg $kg^{-1}$ (Wang *et al*. 2012). A recent review investigating the impact of different types of neonicotinoids at varied concentrations on earthworm survival, reproduction and behaviour was conducted across different types of earthworm species; Pisa *et al*. (2015) concluded that individuals are at risk of mortality if they consume soil or organic particles of about 1ppm for several days. It is possible that long-term low level concentration of neonicotinoids in soil may pose a higher risk to earthworms from sub-lethal effects than can be deduced from short-term toxicity tests, which typically last for a few days (Pisa *et al*. 2015). Here, we experimentally test the effect of field-realistic doses of clothianidin on mortality, weight gain, and food consumption to assess the overall impacts on fitness of chronic exposure of *L. terrestris* individuals.

### 3.3 Methods

*Soil contamination*

The soil moisture content of both a sharp sand and a sterilised Kettering loam was taken using a TDR© 'FieldScout' soil moisture content probe. Kettering loam is used by many researchers as a reliable earthworm culture substrate and has been proposed as a standard medium for toxicology tests (Lowe & Butt 2005), and is sold as pesticide free. The loam was mixed with a sharp sand to make a more friable substrate. Clothianidin stock solution (made up in water) was diluted as appropriate with spring water (ASDA, own brand), then mixed with sand and finally loam to give a 70:30 loam : sand mix with a 25% moisture content (Berry & Jordan 2001).

Treatment groups of 0 ppb (control), 1, 5, 10, and 20 ppb were created, based on concentrations detected in soil collected from conventionally farmed, neonicotinoid treated oilseed rape and winter wheat fields and field margins in the UK (Botias *et al*. 2015). These samples were collected in the spring, approximately 10 months post-drilling of treated crops in fields undergoing conventional arable rotation. 100 ppb was used as a

positive control. While these levels were used to replicate those present up to 40 weeks since seed drilling, it should be noted that levels of 270 to 440 ppb have been found in soil up to three days after a single clothianidin application (Ramasubramanian 2013).

*Food contamination*

Primary waste paper sludge from a paper recycling plant (Sittingbourne, Kent, UK) was mixed with brewer's yeast at a 25:1, carbon to nitrogen ratio following methods described in Butt (1993), and used as a food source (referred to hereafter as "food"). To ensure homogeneous distribution of the clothianidin solution throughout the food, clothianidin stock solution was first added to spring water (ASDA own brand) and yeast before thoroughly mixing in the paper waste. Food was treated to the following levels: 0 ppb (control), 1, 5, 10, 20 and 100 ppb.

*Microcosm set-up*

Tops were removed from 180 4-litre plastic bottles (henceforth described as "microcosms") and they were each filled with 1.5 kg of contaminated soil substrate. Three separate experiments were set up: A – treated soil only, B – treated soil and treated food and C – treated food only, with 10 replicates per treatment group in each exposure and control group. Care was taken to ensure that no large air pockets were present as these could be used by the worms as a refuge. The bottle opening was covered with fine plastic mesh to prevent escape. Every microcosm received 70g of food atop of a stainless-steel mesh (6mm x 6mm) placed on top of the soil substrate. The 720 worms were purchased from Worms Direct (Maldon, Essex, UK) and all were mature with clitellum. Prior to the experiment worms had been fed on leaves but all underwent a 7-day acclimatisation period where their food was swapped to the uncontaminated version of the paper waste and yeast mixture used in this experiment. Each microcosm housed 4 worms. Experiment A received worms that were approximately 2 months older than individuals used to initiate experiments B and C, due to higher than expected mortality in the stock population. Microcosms were kept at 18$^{o}$C, following Lowe & Butt (2005).

*Data collection*

Every four weeks, the contents of each microcosm were emptied into clean buckets, and the worms were gently washed and blotted dry. The worms from each microcosm were weighed together as a group; body mass has previously been shown to be a sensitive biomarker in the earthworm (Dittbrenner *et al*. 2010). In order to avoid additional stress to

the individuals, worm weight was not standardised by voiding the gut contents of individuals prior to worms being weighed. The weight of food remaining on the grill was then subtracted from the starting weight each month and is henceforth described as 'food consumed'. The weight lost from the evaporation of water from the food source was assumed to be constant across replicates, since all replicates were housed in the same environmental conditions. However, it is important to note that some of this food had been taken down into each burrow and stored i.e. it had not actually been ingested by the worms. Cast production can be used as a proxy for earthworm activity (Capowiez *et al*. 2010), however, casts could not be separated from the food as worms had commonly cast directly into their food source. Obvious casts were removed from the edges of the grill before the remaining food was weighed. The worms were then placed back into the bottle with the same soil. The remaining food was discarded and replaced with freshly contaminated food and any water lost through evaporation from the soil (as defined by weight lost from a bottle of soil without worms) over the month was replaced in order to return the soil moisture to 25% (Berry & Jordan 2001). Each experiment ran for four months in total in accordance with the availability of the specialist laboratory space required

*Data analysis*

The average weight of individuals and the average amount of food consumed per worm were calculated every four weeks for each replicate. All analyses were carried out using SPSS version 22 (IBM Corp, 2013). Worm weights across treatment groups were compared using repeated measures ANOVA when assumptions of normality (as defined by the Shapiro-Wilk statistic) were met. The assumption of sphericity (as defined by Mauchly's statistic) was not met for data from any treatment group, therefore Greenhouse-Geisser adjustments were made to correct the ANOVA and it is this adjusted p value that is reported. The within-subject variance of food consumed per worm was found to have significant heterogeneity and therefore non-parametric Kruskall-Wallis H tests were preferred for this variable. Significant effects were investigated further using pair-wise comparisons using Dunn's procedure with a Bonferroni correction for multiple comparisons.

Survival curves were fitted to mortality data for each exposure group using the non-parametric Cox's proportional hazards model (CoxPH). The CoxPH assumes proportional

hazards (chance of mortality) within treatment groups using control group mortality as a reference. The output from this model was compared with a parametric model, alternately assuming a constant hazard and a non-constant hazard with Weibull errors to ensure good model fit (Rotheray, 2012). To assess the effects of treatment on mortality, a separate CoxPH was fitted to compare pooled treatment groups with pooled control groups, applying any level of clothianidin treatment on effect on survival.

### 3.4 Results

*Experiment A: Treated Soil*

Neither the weight or food consumed by worms kept in treated soil and fed untreated food varied significantly across treatment groups over time (weight: *F*= 1.231, D.F= 11.1, p=0.279, repeated measures ANOVA, food: week 4: $X^2(5)= 10.443$, p=0.064; week 8: $X^2(5)=7.073$, p=0.215; week 12: $X^2(5)= 3.817$, p=0.576; week 16: $X^2(5)= 5.44$ p=0.364, Kruskall-Wallis (Fig 3.1 A and B)). Mortality was lowest in the control group across all time points, with 52% of the total population remaining at week 16 (Fig 3.1, C). The CoxPH detected a significant effect of treatment on mortality (Z= 2.348, p=0.0189). However, there was no clear dose-response effect at higher doses (Fig 3.2), with worms exposed to 20ppb clothianidin having the highest mortality (80% by week 16).

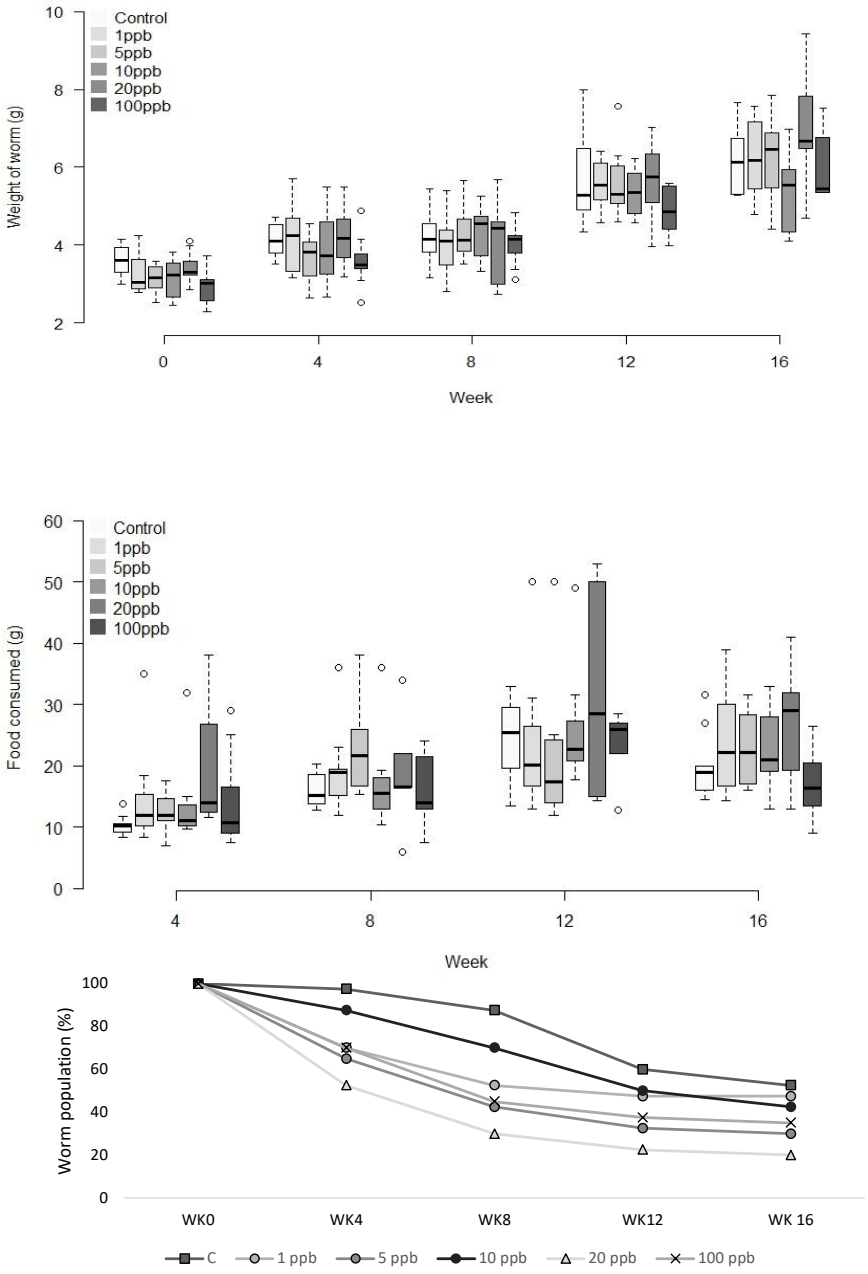



**Figure 3.1** Experiment A: clothianidin treated soil. a) Changes in the mean weight (a) and mean food consumption (b) of *Lumbricus terrestris* individuals over time in clothianidin (CLO) treated soil containing 1, 5, 10, 20, 100 ppb and control. Error bars show standard error of the mean. c) Percentage of worms in relation to initial worm number (n=40 per treatment group).

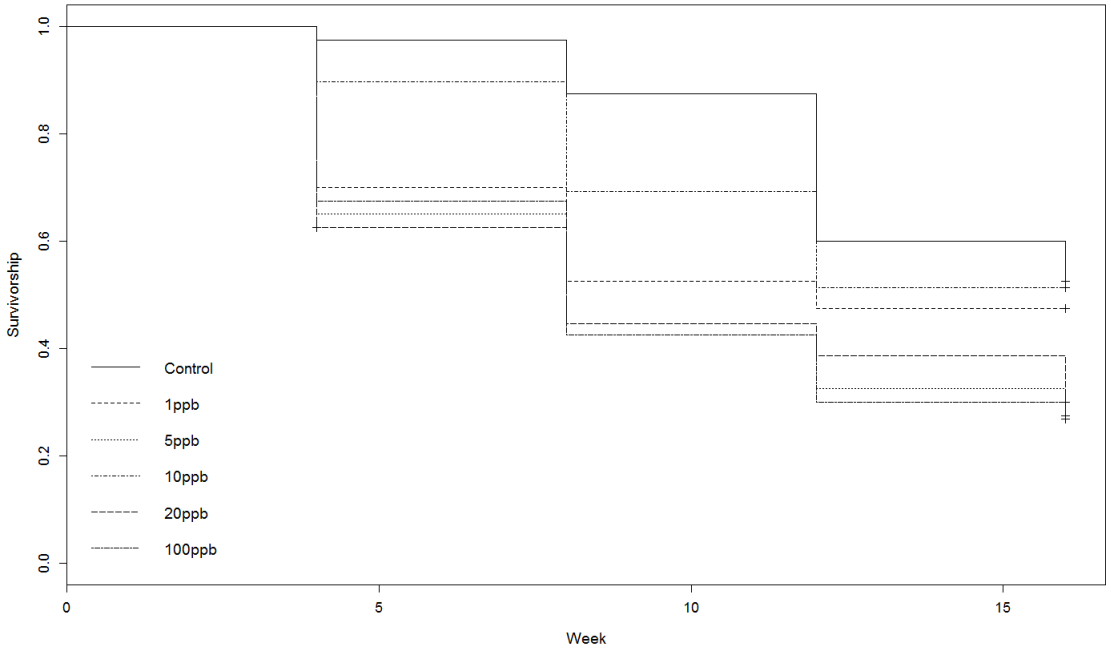



**Figure 3.2** Experiment A: Cox proportional hazards model survival curve illustrating a significant effect of treatment on mortality (Z=2.348, p= 0.0189).

*Experiment B: Treated Soil and Treated Food*

There was no significant difference in the weight of worms between treatments when exposed to clothianidin in both soil and food (Fig 3.3, a) (*F*= 1.825, D.F= 10.365, p=0.062,). Analysis of food consumption revealed significant differences in consumption across treatment groups over time, with generally lower consumption when exposed to higher pesticide concentrations (week 4: $X^2(5)= 29.639$, p≤0.001; week 8: $X^2(5)= 34.876$, p≤0.001 and week 12: $X^2(5)= 11.650$, p=0.040 but not week 16: $X^2(5)= 8.761$, p=0.119). Pairwise comparisons highlight significant differences between all treatment groups and 100ppb (Fig 3.3, B) at week 4 (adjusted p: 1 ppb≤0.001, 5 ppb=0.010, 10 ppb≤0.001, and 20 ppb=0.002), and at week 8 between 100 ppb and 1, 5 and 20 ppb (adjusted p≤0.001, <0.001 and .005, respectively). Highest total mortality was observed in the 100 ppb treatment group (25% mortality) but the differences in mortality between treatment groups was not significant (CoxPH Z = -0.173, p=0.863, Fig 3.3, C).

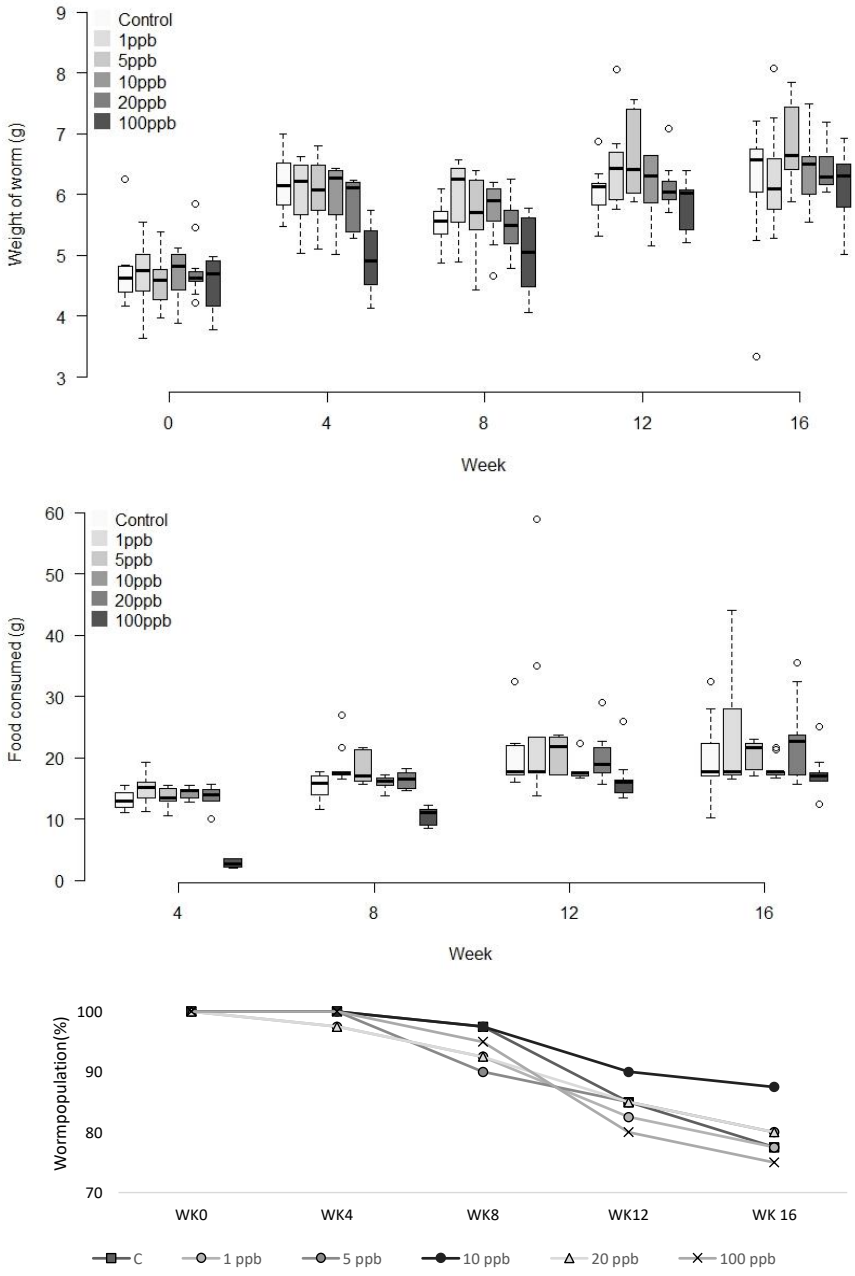



**Figure 3.3** Experiment B: clothianidin treated soil and food. a) Changes in the mean weight (a) and mean food consumption (b) of *Lumbricus terrestris* individuals over time in clothianidin treated soil provided with clothianidin treated food containing 1, 5, 10, 20, 100 ppb and control. Error bars show standard error of the mean. c) Percentage of worms remaining at each time point in relation to initial worm number (n=40 per treatment group).

*Experiment C: Treated Food*

There was no significant relationship between clothianidin concentration and weight for the worms fed on treated food only (*F* = 1.809, D.F = 8.870 p=0.078). However, the amount of food consumed was significantly different across treatment groups at week 4 and week 8 ($X^2$ (5)=35.304, p≤0.001 and $X^2$ (5)= 11.241, p=0.047, respectively). Pairwise comparisons at week 4 show less food being consumed in the 100 ppb group than in other groups bar 20 ppb (adjusted p: 1 ppb≤0.001, 5 ppb≤0.001, 10 ppb=0.004 and Control=0.007), and less food consumed at 100ppb compared to 1ppb (adj. p= 0.039) at week 8. Mortality in Group C (Fig 3.4, C) was highest in worms fed with 20 ppb treated food (27.5% mortality) and lowest in food groups 1ppb and 5ppb (10% mortality). Overall, there was no significant difference in mortality between treatment groups (CoxPH Z = 0.935, p=1.522). It is notable that mortality in experiments B and C was markedly lower than in experiment A which used older worms.

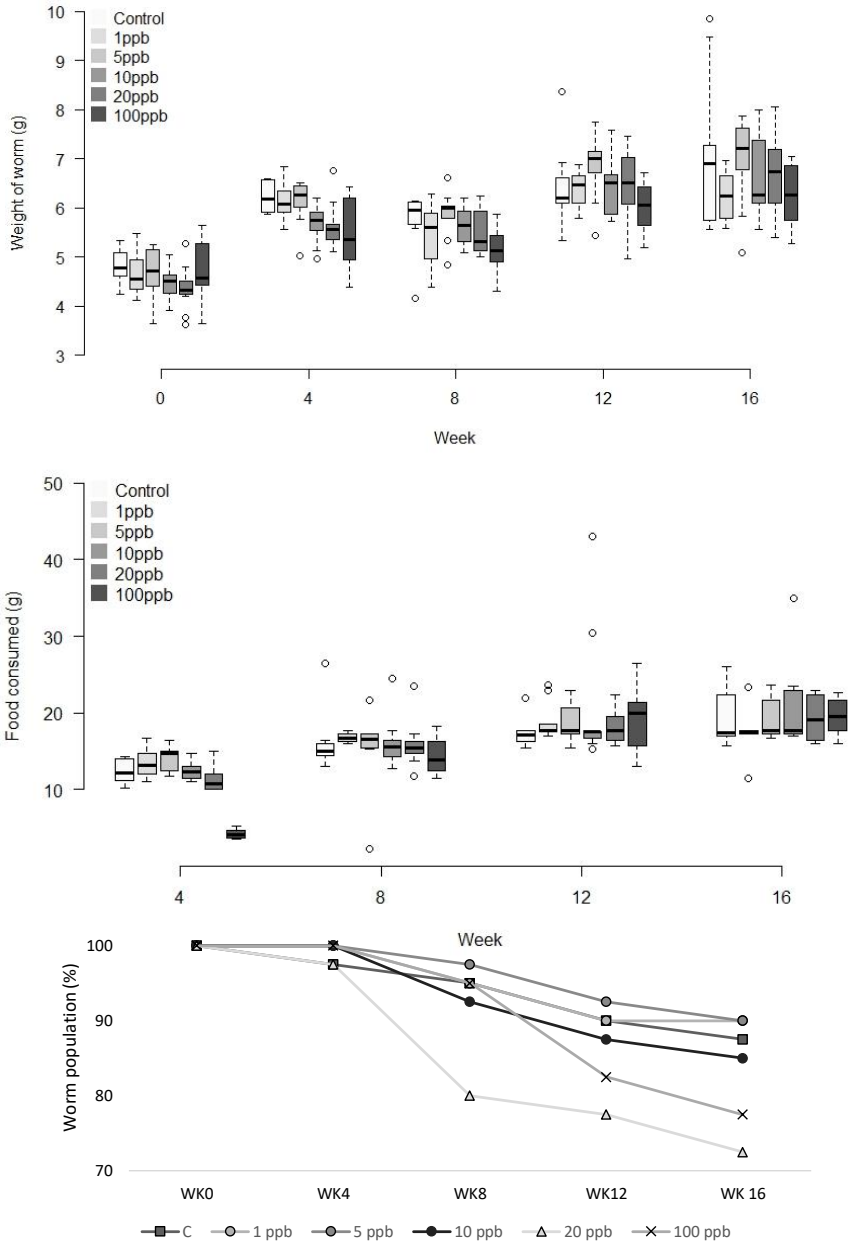



**Figure 3.4** Experiment C: clothianidin treated food. a) Changes in the mean weight (a) and mean food consumption (b) of *Lumbricus terrestris* individuals over time in clothianidin treated soil containing 1, 5, 10, 20, 100 ppb and control. Error bars show standard error of the mean. c) Percentage of worms remaining at each time point in relation to initial worm number (n=40 per treatment group).

## 3.5 Discussion

Our findings suggest that at field-realistic doses, chronic exposure to clothianidin does not have a significant effect on worm weight but that contamination of food can significantly impact the amount of food consumed for up to two months after initial oral exposure, and may also increase worm mortality.

*Mortality*

Mortality levels for worms in experiments with treated food only, and treated food and soil suggest that clothianidin concentrations of ≤ 100ppb do not cause significant mortality above that of the control, whereas there was a significant effect of exposure to treated soil alone, which is somewhat unexpected.

We speculate that this may be because the worms in experiment A were two months older than those in experiments B and C, but this would clearly require further investigation. Patterns of mortality across the three experiments were unclear as all lacked a clear dose-response effect. Of the 13 previous studies on the effects of neonicotinoids on earthworm survival that reported $LD_{50}$ values, only one studied clothianidin but used *E. fetida* as its study species. Wang *et al*. (2012) describe clothianidin as "super-toxic" to *E. fetida* (contact toxicity survival: 0.28 µg/cm, soil toxicity survival: $LC_{50}$=6.06 ppm) though this level is high compared to reported field concentrations and hence the phrase may be misleading. All other studies investigated imidacloprid or thiacloprid and reported $LC_{50}$ ranges from 1.5 to 25.5ppm, with a mean of 5.8 and median of 3.7 ppm (Pisa *et al*. 2015). The longest exposure duration was six weeks, much shorter than the 16 week exposure used in this study. Further, seven of those 13 studies reported lowest effective concentrations ranging from 0.7 to 25 ppm, with a mean of 4.7 and median of 1 ppm (Pisa *et al*., 2015); all of which are concentrations that are an order of magnitude higher the levels used in this experiment.

Our study aimed to test effects of exposure to field-realistic concentrations. Overall, our data suggest that chronic exposure to concentrations of clothianidin up to 100 ppb in food and/or soil have, at worst, only weak effects on mortality of *L. terrestris.* However, it should be noted that our study involved homogeneous mixing of the clothianidin throughout the soil; it is possible that in a real-world situation worms may come across

much higher levels of neonicotinoids by coming into close proximity to treated seeds or applied granules (Pisa *et al*., 2015).

*Weight*

The presence of contaminants in soil may cause stress to the individual which can divert energy from reproduction, burrowing activity and growth (Pelosi *et al*. 2014). The use of body mass change as a biomarker is thought to be ecologically relevant, as high losses in body mass are thought to lead to negative effects on survival and reproduction (Dittbrenner *et al*. 2010). We found clothianidin to have no significant impact on body mass even over 16 weeks of exposure. Body mass in earthworms has not been used as a measured end point in experiments with clothianidin but comparison can be made with other neonicotinoids (Pisa *et al.*, 2015). Three studies have monitored sub-lethal effects of imidacloprid on body mass and weight change in *L. terrestris* with lowest effective concentrations at 0.66 ppm (Dittbrenner *et al*., 2010), 0.189 ppm (Capowiez *et al*., 2010) and 2ppm (Dittbrenner *et al*., 2011), all of which are higher than the treatments used in this experiment and above those generally found in the field. Our data suggest that field realistic exposure of *L. terrestris* to clothianidin does not impact on body mass.

As guts were not voided before weighing, it is possible that differences in worm weight between treatments could be masked or exaggerated by differences in gut content, for example if anti-feedant effects at high doses reduced gut content. However, we would expect this to lead to lower apparent mass at higher doses, which was not detected.

*Food consumption*

In this study we found clothianidin to have a significant negative effect on food consumption for the first 2 months of the experiment in groups where both the soil and food was contaminated and where only food was contaminated. We cannot discern whether the worms were able to detect and were repelled by the pesticide, or whether consumption reduced their subsequent appetite. A previous study with a different worm species, *Apporectodea spp.*, has shown that field-rate application of clothianidin (applied at 0.15 kg/ha) can retard long-term (four months) grass clipping decomposition (Larson *et al*. 2012), a finding which our study corroborates. Reduced decomposition could potentially have long-term impacts on soil organic matter content which may be detrimental to crop growth.

An interesting feature of our data is the recovery of feeding rates towards the end of the experiment. As newly spiked food was provided every four weeks, this recovery is not because of a breakdown of clothianidin; it may be because the worms became desensitised, or because feeding inhibition was overridden by hunger. Food consumption recovered more quickly when only food was contaminated, compared to when both soil and food were contaminated, suggesting that both oral and contact exposure retards the recovery of the individual to a greater degree than oral exposure alone. Oligochaetes have been found to increase digging activity when exposed to thiamethoxam (Alves *et al*. 2013), so it is possible that any negative effect of exposure to clothianidin through treated soil is masked due to an irritant effect: a worm's energy requirement may increase as a result of elevated activity caused by irritation, which may therefore increase food consumption.

Earthworms are known to be able to distinguish pollutants in soil, though it is not known if this behaviour is due to being able to discern the biological availability of pollutants or other factors (Alves *et al*. 2013). Our study design meant that individuals were unable to avoid the contaminated soil, and therefore laboratory exposure duration may not be representative of a typical field exposure duration; in a field-realistic scenario the individuals might be able to move away from contaminated soil, even though full field application of neonicotinoids is the norm. For example, they may be able to burrow deeper where contamination is likely to be lower. In this respect our experimental design may exaggerate effects compared to real-world situations. On the other hand, the results from this single chemical exposure experiment may not adequately reflect the full effect of the contaminant on *L. terrestris* as in field conditions they may often encounter multiple pesticides. Previous work on the impact of insecticidal chemistries on beneficial non-target arthropods and earthworms has shown there to be more significant effects from exposure to combination products than the singular components alone (Larson *et al*, 2012).

The effects of neonicotinoid pesticides are often discussed assuming that all neonicotinoids act in the same way with regard to their target sites and their effects. However, individual neonicotinoids have been reported to have distinct binding to the nicotinic acetylcholine receptor (nAChRs) and therefore may pose differential risks to non-target organisms (Moffat *et al*., 2016). It would thus be unwise to assume that other neonicotinoids would have similar effects on earthworms to those that we describe for clothianidin (Moffat *et al*. 2016, Dittbrenner *et al*. 2011).

Our results show that chronic exposure of *L. terrestris* individuals to clothianidin at concentrations up to 100 ppb has no significant long term effect on the weight of individuals but has significant negative impact on the amount of food consumed over a 2-month period. We also found some evidence of elevated mortality at higher doses in older worms. The eventual recovery of food consumption exhibited in individuals fed treated food highlights the importance of long-term chronic exposure studies; previous experiments have only tested the acute effects of neonicotinoid pesticides on *L. terrestris*, and have tended to use very high doses that may not commonly occur in the field. Although we cannot rule out negative effects on worms over longer periods, when in the immediate vicinity of treated seeds, or from combined exposure to neonicotinoids and other pesticides or stressors, our results suggest that exposure to soils and foodstuffs contaminated with field-realistic levels of clothianidin does not have lasting harmful effects on the growth or survival of younger *L. terrestris* individuals. Further work is required to investigate how worm age may influence their susceptibility to pesticides.

# **Chapter 4** - Neonicotinoids Thiamethoxam and Clothianidin Adversely Affect the Colonisation of Freshwater Microcosms.

This chapter has been published as:

Basley, K., and Goulson,D (2018), Neonicotinoids Thiamethoxam and Clothianidin Adversely Affect the Colonisation of Freshwater Microcosms. Environmental Science and Pollution Research. doi.org/10.1007/s11356-017-1125-5

KB and DG conceived the study and were responsible for study design. KB collected the data. KB and DG analysed the data and wrote the manuscript.

## 4.1 Abstract

Surface waters are sometimes contaminated with neonicotinoids: a widespread, persistent, systemic class of insecticide with leaching potential. Previous ecotoxicological investigations of this chemical class in aquatic ecosystems have largely focused on the impacts of the neonicotinoid imidacloprid; few empirical, manipulative studies have investigated the effect on invertebrate abundances of two other neonicotinoids which are now more widely used: clothianidin and thiamethoxam. In this study, we employ a simple microcosm semi-field design, incorporating a one-off contamination event, to investigate the effect of these pesticides at field-realistic levels (ranging from 0 to 15 ppb) on invertebrate colonisation and survival in small ephemeral ponds. In line with previous research on neonicotinoid impacts on aquatic invertebrates, significant negative effects of both neonicotinoids were found. There were clear differences between the two chemicals, with thiamethoxam generally producing stronger negative effects than clothianidin. Populations of Chironomids (Diptera) and Ostracoda were negatively affected by both chemicals, while Culicidae appeared to be unaffected by clothianidin at the doses used. Our data demonstrate that field-realistic concentrations of neonicotinoids are likely to reduce populations of invertebrates found in ephemeral ponds, which may have knock on effects up the food chain. We highlight the importance of developing pesticide monitoring schemes for European surface waters.

## 4.2 Introduction

The majority of species in freshwater aquatic ecosystems are arthropods. These are an essential link in the transfer of energy up the freshwater food chain, being a primary food source for many species of vertebrates, such as fish, amphibians and birds (Chagnon *et al*., 2015). A decrease in arthropod abundance or diversity is therefore likely to result in a loss of important ecosystem processes and knock-on effects for higher trophic levels (Covich *et al*. 2004; Hallmann *et al*. 2014).

Small-scale aquatic habitats such as temporary ponds and puddles often fulfil an important ecological role at the landscape level (De Meester *et al*., 2005). Similarly, ditches are crucial features for land drainage and, if managed properly, can also provide habitats for wildlife. Although such ephemeral habitats are the least species rich of the freshwater features in an agricultural landscape, they have been found to support a diversity of specialist temporary water invertebrates (Williams, 2004). Nicolet *et al*. (2004), found that, of 71 temporary ponds

surveyed in England and Wales, 75% of these supported at least one nationally scarce macro-invertebrate and 8% supported at least one nationally scarce plant species across a range of physico-chemical characteristics.

Globally, neonicotinoids have become the most widely used insecticides due in part to their systemic properties in the crop to be protected and also their relatively low vertebrate toxicity (Jeschke *et al*., 2011). However, with the exception of the Netherlands, most countries in Europe and other parts of the world do not have a system in place for the monitoring of neonicotinoid pesticides in aquatic systems, although the monitoring of pesticide presence in water is required under the European Drinking Water Directive (Allan *et al*., 2006). It has been shown that at the global scale, more than 50% of detected insecticide concentrations exceed regulatory levels, indicating that surface waters and therefore aquatic biodiversity, are at risk of harm from current insecticide use (Stehle and Schulz, 2015). In the UK a two metre protection zone must be left around ditches and watercourses in all fields of two hectares or more to minimise water contamination (DEFRA, 2006). However, the risk of contamination via neonicotinoid seed dressings is not currently addressed; the only stipulation in their use is that treated seeds are kept away from surface water, which does not account for the possibility of lateral movement of neonicotinoids through the soil profile, nor movement of the pesticide in surface run-off.

There are widespread concerns as to their potentially far-reaching impacts upon wildlife (Chagnon *et al*., 2015; Goulson, 2013; Hallmann *et al*., 2014; Pisa *et al*., 2015; Van Dijk *et al*., 2013; Whitehorn *et al*., 2012). Neonicotinoids and their toxic metabolites have been found to be persistent, not just in the target plant, but also in water, aquatic sediments and soil (Van der Sluijs *et al*., 2013). A recent review concluded that low levels of neonicotinoids cause negative effects on aquatic ecosystems both at the individual and population level (Pisa *et al*., 2015), and the effect has been found to extend to zooplankton, benthic and neuston communities (Hayasaka *et al*., 2012).

The persistence of neonicotinoids increases the duration over which non-target organisms may be exposed (Krupke *et al*. 2012, Van der Sluijs *et al*. 2013). Where the neonicotinoid is used as a seed dressing, studies have shown that only 1.6 – 20% of the active ingredient is absorbed by the crop. The remainder is either lost as dust during sowing (approximately 1-2%) or enters the soil (typically more than 90%) (Tapparo *et al*., 2012). Due to their high run-off and capacity to leach into surface and ground waters (González-Pradas *et al*., 2002), neonicotinoids have often

been detected in aquatic environments, including streams, lakes and temporary bodies of water such as puddles (Chagnon *et al*., 2015).

Imidacloprid, one of the earlier most widely used neonicotinoids has been found in the Netherlands in groundwater, streams and ditches at concentrations far exceeding the maximum allowable risk level (13ng/l) and has also been detected in 89% of rivers, creeks and drains in California, 19% of those samples exceeding the US Environmental Protection Agency (EPA) guideline concentration of 1.05ppb (Starner and Goh, 2012). However, it is common for residue levels of neonicotinoids to be much lower; a survey of surface water contamination studies found clothianidin to be generally in the region of 0.003 – 3.1ppb and thiamethoxam to be around 0.001 - 225 ppb (Morrissey *et al*., 2015). Surface water, including puddles, ditches, irrigation channels and streams in or near farmland have been found to be contaminated by neonicotinoids (Morrissey *et al*. 2015; Van Dijk *et al*. 2013; Samson-Robert *et al*. 2014, Main *et al*. 2014, Schaafsma *et al*. 2015). Contamination levels of various types of surface waters differ. For example, samples taken from within and around the perimeter of corn fields in Southwest Ontario detected residues of clothianidin (mean = 2.28 ppb, maximum = 43.60 ppb) and thiamethoxam (mean = 1.12 ppb, maximum = 16.50 ppb) in 100% and 98.7% of samples tested, respectively (Morrissey *et al*., 2015; Schaafsma *et al*., 2015). Streams near to fields of corn and soybean production contained median levels of 8.2 ppb of clothianidin and levels of <2ppb thiamethoxam (Hladik *et al*., 2014). Both thiamethoxam and clothianidin have relatively long half-lives in soil: the DT50 of clothianidin is 148 – 1,155 days, and thiamethoxam's is 229 days on average, and the effects of temperature, sunlight exposure, soil water balance and soil type can greatly affect the half-life (Main *et al*., 2014). Their persistence in the soil and high water solubility (thiamethoxam =4,100 mg/L; clothianidin =327 mg/L (Main *et al*., 2014)) means there is high potential to be transported into surface waters.

A significant negative relationship between imidacloprid-polluted surface water and macro-invertebrate abundance has been found, after accounting for land-use differences between sites (Van Dijk *et al*., 2013). The authors found that macro-fauna abundance dropped off sharply between 0.013 ppb and 0.067 ppb imidacloprid, concentrations more than an order of magnitude below the EPA guidelines. The results of an extensive review of laboratory and semi-field microcosm studies indicates that aquatic invertebrates are highly sensitive to neonicotinoids (Pisa *et al*., 2015). However, most of the studies were conducted using imidacloprid, a compound that is now relatively little-used (Goulson, 2013), having been largely replaced by clothianidin or thiamethoxam (DEFRA 2014). There is thus a need to further

investigate the impacts of these newer neonicotinoids on aquatic ecosystems. Here, we experimentally test the effect of field-realistic doses of clothianidin and thiamethoxam on the colonisation and development of aquatic invertebrate populations in puddle-replicate microcosms in semi-field conditions.

## 4.3 Methods

*Microcosm Set-up*

Temporary water bodies were simulated by filling new 14 litre plastic buckets with 400g of loamy soil and 10L of either untreated or treated water (henceforth described as "microcosms"). The relative simplicity of the microcosm design allows temporary aquatic ecosystems to be created with high levels of replication (De Meester *et al*., 2005). Soil was collected from a single site, with no recorded history of neonicotinoid usage, on the University of Sussex campus on the 20th August 2014. The soil was thoroughly mixed using a clean spade before being divided into 400g samples which were placed in the clean buckets, these were left in the laboratory overnight.

In total, 140 microcosms were created on the 21st August 2014; 20 were controls, while ten microcosms were used for each of the following concentrations: 0.1, 1, 3, 7, 10 and 15 ppb of either thiamethoxam or clothianidin. Stock solutions were produced from analytical grade clothianidin and thiamethoxam (Sigma-Aldrich, Gillingham, UK) and made up in deionised water as they did not need to be stored. The concentrations of 0.1, 1 and 3 ppb were used to replicate levels that may be present in surface water due to chronic contamination after rain fall and leaching. The concentrations of 7, 10 and 15 ppb were used to replicate a singular pulse contamination i.e. a rainfall event immediately after the sowing of a treated crop, before the active compound has bound to soil particles. Concentrations of clothianidin and thiamethoxam used in this experiment were within the ranges detected in a review of surface water samples (Morrissey *et al*., 2015). The buckets were filled with 10 L of fresh tap water and then dosed with neonicotinoid to create the contaminated microcosms. Once dosed, the soil and water fraction were thoroughly mixed. The microcosms were placed immediately adjacent to one another on a strip of grassland between two buildings in a 28 x 5 randomised block, and were left uncovered to allow for colonisation by flying insects. This meant that the microcosms were subject to rainfall but this did not lead to overflow, and no one microcosm was subject to more rainfall than another. Microcosms were left *in situ* for 33-38 days.

*Data collection*

As the microcosms had been filled to 10L with fresh tap water and dosed straight after set-up, it was expected that the population of aquatic invertebrates in the microcosms at the start of the experiment would be zero. The ostracoda subsequently detected in the microcosms were likely to have been present as eggs in the soil, but were assumed to be evenly distributed as a result of the thorough mixing at the set-up stage.

To reduce disturbance in the aquatic microcosms, sampling of the invertebrate populations was undertaken completely at the end of the experimental period, rather than taking periodic samples throughout. Commencing on 23rd September 2014, the invertebrate composition of the microcosms was quantified over a 5-day period, in a random order (using a random number generator). The water fraction was slowly poured through rinsed muslin in order to collect the live aquatic organisms that remained at the end of the experimental period; these were then stored in ethanol. The soil was rinsed through a 2 mm sieve to remove the larger stones and collected in a 250 µm sieve underneath in 100 g sub-samples to allow thorough searching for invertebrates. To collect the Chironomids, the sieve with the soil sample was slowly submerged so that Chironomid larvae floated to the surface; these were collected in a small hand-held sieve and stored in the ethanol.

The samples were subsequently drained through a 125 µm sieve to separate the organisms from the ethanol; organisms were then rinsed with deionised water. The sample was placed onto a white plastic tray marked with a grid, a small amount of water was added and the tray was gently shaken to distribute the sample across the grid. The organisms present were identified and counted by eye. Identification was to subclass for aquatic mites (Acari), order for Ostracoda and family for Chironomidae and Culicidae. After counting, the sample was retained in ethanol for reference. Two control microcosms were lost due to problems during sampling.

*Statistical Analysis*

All statistical analyses were conducted using IBM SPSS Statistics 22. The control replicates were pooled. Non-parametric Kruskal-Wallis H tests were preferred due to the significant heterogeneity found across the four organisms' abundance data (too few Acari were detected for statistical analysis). These were used to test for significant differences across ranked means in the four populations (Chironomidae, *Culex* larvae, *Culex* pupae and Ostracoda) between groups of 7 concentrations (control n=18, 0.1, 1, 3, 7, 10 and 15 ppb all n= 10.). Post-hoc

Dunn's tests with Bonferroni correction were used to determine significant differences between concentrations for each concentration group and for each neonicotinoid.

### 4.4 Results

Invertebrate populations in the microcosms contaminated with thiamethoxam showed significant differences across concentrations, with a general pattern of reduced numbers at higher concentrations of insecticide (Figure 4), apart from *Culex* larvae whose numbers were highest at both the lowest and the highest concentrations. Ostracod numbers tended towards greater abundance in the low concentrations, with the greatest numbers being found in the control group; pairwise comparisons showed a significant difference between the control and 0.1 ppb and 15 ppb (adj. p = 0.033 and 0.029 respectively).

Chironomidae, *Culex* pupae and Ostracoda showed a significant response to clothianidin concentration (Figure 4), yet patterns for clothianidin were a less clear than for thiamethoxam. For Chironomidae, the lowest abundance was found at the three highest clothianidin concentrations, with significant pairwise relationships between the control and the two highest concentrations (10 ppb adj. p= 0.048, 15 ppb adj. p= 0.003). Interestingly for clothianidin, low concentrations (0.1, 1 and 3 ppb) supported more Ostracod individuals than the controls, a pattern not replicated for thiamethoxam. A significant difference was noted between 1 ppb and 15 ppb (adjusted p = 0.023). *Culex* larvae exhibited no statistically significant relationship between concentration and abundance.

**Figure 4**

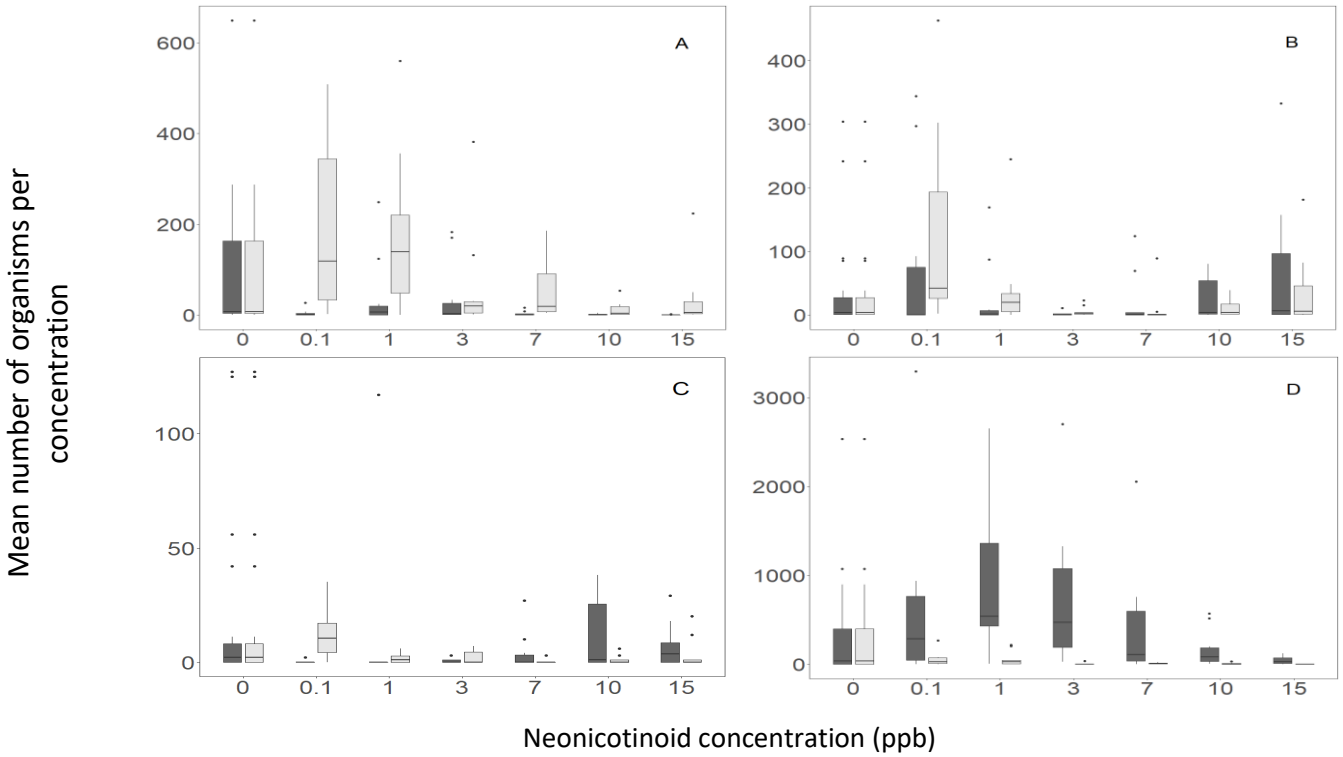



**Figure 4.** Effect of thiamethoxam (light grey) and clothianidin (dark grey) on mean population of aquatic invertebrates, for each separate neonicotinoid, means labelled A are significantly different than those labelled B; Dunn's with Bonferroni correction. A-Chironomidae: thiamethoxam ($X^2$ (6)= 16.1, p=0.013)); 0.1ppb -10 adj. p= 0.036, 1ppb – 10ppb adj. p= 0.048; clothianidin ($X^2$ (6)= 21.9, p= 0.001)); control – 10ppb adj. p= 0.048, control – 15ppb adj. p= 0.003). B- Culex larvae: thiamethoxam ($X^2$ (6)= 20.8, p= 0.002)); 0.1ppb- 3ppb adj.p = 0.031, 0.1ppb – 7ppb adj. p= 0.001; clothianidin – no statistically significant relationship existed between concentration and population abundance despite numbers dipping at 3 and 7ppb (p= 0.498). C – Culex pupae: thiamethoxam ($X^2$ (6)=14.8, p= 0.021)); 0.1ppb- 7ppb adj. p= 0.021; clothianidin ($X^2$ (6)=14.5, p= 0.025)); despite a statistically significant relationship overall , post-hoc results showed no overall difference between means of each concentration replicate group when examining adjusted significance. D – Ostracoda: thiamethoxam ($X^2$ (6)=20.46, p= 0.002)); control – 15ppb adj. p = 0.033, 0.1ppb -15ppb adj p= 0.029; clothianidin ($X^2$ (6)=17.6, p= 0.007)); 1ppb – 15ppb adj. p= 0.023.

## 4.5 Discussion

Our data show that field-realistic concentrations of two commonly used neonicotinoids, thiamethoxam and clothianidin, significantly impact on the populations of invertebrates (Diptera and Ostracoda) that colonise aquatic microcosms, with some differences between the effects of the two chemicals. The aquatic microcosms were colonised mainly by flying Diptera (*Culex* and Chironomidae) which oviposited in the water, and also by Ostracoda, which may have originated from the soil added to each microcosm (they can survive for long periods in soil as desiccation-resistant eggs) (Özuluğ & Suludere 2012)). Organisms were found to differ in their sensitivity to both the concentration and particular class of neonicotinoid.

In a review of 214 toxicity tests including acute and chronic tests for neonicotinoids, Chironomidae were amongst the most sensitive taxa with many species exhibiting short-term lethal effects at clothianidin water concentrations of 1-29 µg/l (EC 2005 Summary; reviewed in Morrissey *et al*., 2015). A significant effect of thiamethoxam was observed on *Culex* pupae and *Culex* larvae; the relationship for *Culex* larvae was absent in the clothianidin microcosms which could be due to the higher concentrations of clothianidin delaying the development of the larvae, this effect has also been found in *C. riparious* exposed to thiamethoxam (Saraiva *et al*., 2017). Work by Sanchez-Bayo and Goka (2006) found that for the three freshwater Ostracod species investigated, 48h $LC_{50}$ was in the range of 301-715 µg/L for imidacloprid, far higher than the levels used here. However, the immobilisation bioassays for the same species were calculated to be in the range of 11-22 µg/L (24h) and 5-7 µg/L (48hr), and clearly if such sub-lethal effects occurred in our microcosms then we would also expect impairment of feeding and reproduction due to the similar toxicity levels for aquatic organisms and identical mode of action of neonicotinoids (Morrissey *et al*., 2015).

It is possible that the actual final concentrations of neonicotinoids to which invertebrates were exposed in our microcosms were lower than those with which the water was originally dosed. Neonicotinoids are subject to rapid photolysis in clear water and our microcosms were placed in a well-lit position in late summer. However, toxicity tests for imidacloprid performed under light or dark conditions have shown that $LC_{50}$ values were not significantly different for any of the ostracod or cladoceran species tested; there is evidence to suggest that photolytic half-lives are difficult to relate to the actual persistence of neonicotinoids in natural waters (Sánchez-Bayo and Goka, 2006), but our results should be interpreted with this caveat in mind.

Of course, degradation of pesticides following pulse contamination events would be expected in real water bodies in the field, so in this sense our microcosms are field-realistic.

The microcosms were also open to rainfall which would have diluted the pesticides, as it would concentrations in natural puddles. However, neonicotinoids persist for much longer in soils, so it is likely that they persisted in the soil fraction of the microcosm habitat. The difference in soil affinity of the two compounds could explain some of the observed differences in response between organisms (Morrissey *et al*., 2015). It is possible that after contamination with the pesticides, clothianidin bound to the soil fraction of the microcosm to a greater degree than thiamethoxam, and therefore less clothianidin was active in the water fraction. It is also possible that the more rapid photolysis of clothianidin (Morrissey *et al*., 2015) might have reduced its concentration in the water to a greater degree than that of thiamethoxam, potentially explaining the absence of a measurable effect on *Culex* larvae*,* which inhabit the open water. It is important to note that thiamethoxam degrades to clothianidin, so organisms are exposed to the toxic mixture for longer because the parent compound (thiamethoxam) is more stable in water, while the metabolite clothianidin is more persistent in soil (Morrissey *et al*., 2015); so the overall exposure is longer than if the organisms were only exposed to clothianidin.

It should be noted that our study does not attempt to distinguish between effects of the pesticides on colonisation of the microcosms, and subsequent toxicological impacts on invertebrates. Reduced numbers of dipteran larvae could be due to either of these processes as Easton and Goulson (2013) report avoidance of pan-traps containing solutions of imidacloprid well below 1ppb by dipterans. However, Ostracoda do not fly and it seems likely that they were in the soil placed into the buckets at the beginning of the experiment. The significant relationship between Ostracod number and increasing thiamethoxam and clothianidin concentration is therefore likely to be due to the toxicity of the compounds and not to any avoidance behaviour exhibited by this invertebrate.

Our data corroborate previous studies which suggest that neonicotinoids are likely to be broadly impacting aquatic invertebrates (Main *et al*., 2016; Mohr *et al*., 2012; Pestana *et al*., 2009a, b). All previous mesocosm studies of this nature have studied the impacts of imidacloprid; we show that a single contamination at time-zero of a novel temporary water body by field-realistic levels of either thiamethoxam or clothianidin has a detrimental effect on the development of invertebrate populations, and invertebrates already present in the soil.

Van Dijk *et al*. (2013) describe broad patterns of reduced abundance of aquatic invertebrates in the Netherlands in permanent aquatic habitats where imidacloprid concentrations exceeded 13 ng/L. Such an effect has the potential to change the structure of the food web by affecting the population levels of the base organisms and therefore the transfer of energy to consumers (Chagnon *et al*., 2015). The knock-on, and potential cascading effects of a neonicotinoid presence in freshwater have been indicated by Hallmann *et al*. (2014), who demonstrated that depletion of insect food resources caused by pollution of aquatic habitats had a negative impact on insectivorous passerine bird species in the Netherlands. Areas where imidacloprid concentrations in surface water were more than 20 ng/L saw the bird population decline by an average of 3.5% annually, for a period of 20 years. The invertebrates that inhabit temporary ponds are also an important food for vertebrate predators such as bats and birds, so our data add to the growing evidence that pollution of aquatic habitats may be contributing to cascading impacts on higher trophic levels**.** The data collected in this study further emphasises that there is a clear and pressing need for more extensive monitoring of pollution of aquatic habitats with neonicotinoids to allow us to properly evaluate the scale of this threat.

# **Chapter 5** - Effects of field-relevant concentrations of clothianidin on larval development of the butterfly *Polyommatus icarus* (Lepidoptera, Lycaenidae)

This chapter has been published as:

Basley, K., and Goulson, D. (2018), Effects of field-relevant concentrations of clothianidin on larval development of the butterfly *Polyommatus icarus* (Lepidoptera, Lycaenidae). Environmental Science & Technology, 52, 7, 3990-3996. DOI 10.1021/acs.est.8b00609

KB and DG conceived the study and were responsible for study design. KB collected the data. KB and DG analysed the data and wrote the manuscript

## 5.1 Abstract

Arable field margins are often sown with wild flowers to encourage pollinators and other beneficial or desirable insects such as bees and butterflies. Concern has been raised that these margins may be contaminated with systemic pesticides such as neonicotinoids used on the adjacent crop, and that this may negatively impact beneficial insects. The use of neonicotinoids has been linked to butterfly declines, and species such as the common blue butterfly (*Polyommatus icarus*) that feed upon legumes commonly sown in arable field margins, may be exposed to such toxins. Here, we demonstrate that the larval foodplants of *P. icarus* growing in an arable field margin adjacent to a wheat crop treated with the neonicotinoid clothianidin, not only contain the pesticide at concentrations comparable to and sometimes higher than those found in foliage of treated crops (range 0.2 to 48 ppb), but remain detectable at these levels for up to 21 months after sowing the crop. Overall, our study demonstrates that non-target herbivorous organisms in arable field margins are likely to be chronically exposed to neonicotinoids. Under laboratory conditions, exposure to clothianidin at 15 ppb (a field-realistic dose), or above, reduced larval growth for the first 9 days of the experiment. Although there was evidence of clothianidin inducing mortality in larvae, with highest survival in control groups, the dose-response relationship was unclear. Our study suggests that larvae of this butterfly exhibit some deleterious sub-lethal and sometimes lethal impacts of exposure to clothianidin, but many larvae survive to adulthood even when exposed to high doses.

## 5.2 Introduction

British butterfly and moth species have been in steep decline since formal recording began in 1976, with 70% of species declining in occurrence, and 57% declining in abundance (Fox *et al*., 2015). Species found on farmland appear to be faring particularly badly with nearly a 40% decline since 2005 in the abundance of English butterfly species alone, despite significant conservation investment in agri-environment schemes (Fox *et al*., 2015; Van Swaay *et al*., 2015; Gilburn *et al*., 2015). Similar patterns of decline have been observed around the globe, at least in locations where populations are accurately monitored (Butchart *et al*. 2010). The intensification of farming over the last few decades has led to a loss of habitat for wildlife (Chamberlain *et al*., 2000), and given that greater numbers of butterflies and butterfly species have been found on organic farms compared to conventional farms (Feber *et al*., 1997; Rundlöf *et al*., 2008), this suggests that chemical inputs are contributing to declines of Lepidoptera. However, as organic farms may have a greater abundance and diversity of food

plants (Feber *et al*., 1997), and greater species richness caused by an increase in landscape diversity (Rundlöf *et al*., 2008), the contribution of chemicals may not be the sole difference between the two farm types.

Neonicotinoid pesticides, alongside glyphosate, currently dominate the global pesticide market, and are used more than any other class of insecticides (Jeschke *et al*., 2011). The current prophylactic use of neonicotinoids as seed dressings on many arable crops (Lundin *et al*., 2015), combined with their persistence, solubility in water and systemic action in plants, presents a large-scale risk of neonicotinoid contamination of non-target plants. This includes the vegetation, pollen and nectar of semi-natural areas surrounding arable crops (Jones *et al*., 2014; Botías *et al*., 2015; Botías *et al*., 2016), and hence poses a risk to non-target organisms living in these areas. In recent years, the majority of attention on neonicotinoid toxicity has been focussed on the risks to bees, as these are economically important pollinators of food crops. Semi-field and laboratory studies suggest that exposure of adult bees to field-relevant doses can impair pollen collection, increase worker mortality, reduce the production of new queens and weaken the bee's immune system (Gill & Raine., 2014; Gill *et al*., 2012; Whitehorn, *et al*., 2012; Di Prisco, *et al*., 2013). However, this gives little insight into the impact of these compounds on other non-target groups. Research into the effect of neonicotinoids on Lepidoptera has focussed on their effectiveness against moth larvae typically regarded as pests (reviewed in Pisa *et al*. (2015). These studies have found a wide range of interspecific tolerances to pesticides, with some moth species up to 100 times more sensitive than others (Stará & Kocourek, 2007). Of the few studies that have looked at the impact of neonicotinoids on non-target Lepidoptera, the focus has been on the effects of acute exposure on larvae (Pecenka & Lundgren, 2015; Yu *et al*., 2016), though in the field we would expect moth and butterfly larvae to be exposed throughout the duration of their development (Botías *et al*., 2016).

Two recent studies found neonicotinoid use to be significantly negatively correlated with long-term butterfly population declines, both in the UK (Gilburn *et al*., 2015) and in California (Forister *et al*., 2016), though it is not clear whether this is due to larval exposure, adult exposure, or both. Relatively few data exist providing field-relevant contamination levels in non-target vegetation**,** but those studies that do (Botías *et al*., 2015, Krupke *et al*., 2012; Greatti *et al*., 2006; Rundlöf *et al*., 2015; Stewart *et al*., 2014), show that neonicotinoid concentrations overlap with lethal concentrations for some insect species (Botías *et al*., 2016).

A recent review has suggested that there is a clear need for studies on the impact of neonicotinoids on non-target butterflies (Pisa *et al*., 2015), in particular those species, such as *Polyommattus icarus* (Lycaenidae, Rottemburg, 1775), which inhabit agricultural landscapes and are at risk of exposure to neonicotinoids (Botías *et al*., 2016). Here, we seek to address this gap in knowledge by first establishing typical vegetative contamination levels of *P. icarus'* food plants in the field, and second experimentally testing the effect of field-realistic doses of clothianidin via oral exposure on the mortality and development of *P. icarus* larvae.

## 5.3 Methods

*Field Study*

*Environmental sampling*

The field study was based at Hope Farm, Cambridgeshire, UK, and was carried out over a 2-year period from September 2013 to August 2015. Prior to this study these fields had never been exposed to neonicotinoids either through direct soil treatment or the growing of neonicotinoid treated crops. In September 2013 Field A was planted with untreated winter wheat and Field B was planted with winter wheat treated with Redigo Deter© (active ingredients: 50 g/L prothioconazole and 250 g/L clothianidin (Botías *et al*., 2015)). No pollen and wildlife margins were created at this point as both fields were planted to their edges with wheat. In September 2014, a field margin of two meters was created around both fields and planted with sainfoin *Onobrychis viciifolia*, winter vetch *Vicia villosa*, birdsfoot trefoil *Lotus corniculatus*, Lucerne *Medicago sativa*, alsike clover *Trifolium hybridum* and dwarf amenity ryegrass *Lolium perenne*, to create a pollen and nectar margin. In October 2014, Field A was sown with neonicotinoid treated wheat and Field B with untreated wheat. Therefore, the margin of Field A would be growing alongside a treated crop and Field B's margin was planted in contaminated soil from the treated wheat planted in the first year. Thus, the margins were sampled 10 and 21 months after neonicotinoid exposure for Field A and Field B respectively. Figure 5.1 summarises the planting schedule.

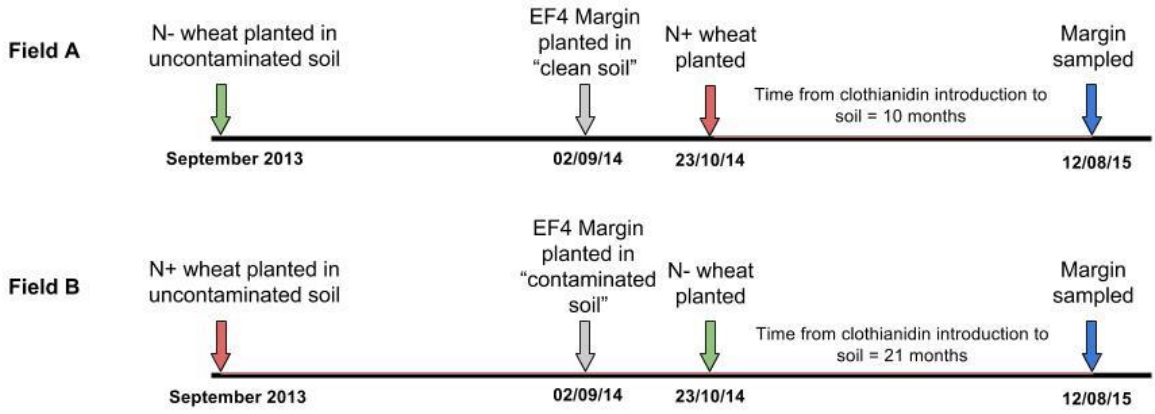



**Figure 5.1** Timings of the planting and harvest of untreated and clothianidin treated winter wheat, and vegetation sampling dates at Hope Farm, Cambridgeshire. N+ indicates a clothianidin seed treatment, N- indicates untreated seed.

On 12th August 2015, twenty samples of *Lotus corniculatus* and *Trifolium hybridum* (Fabaceae), both common larval foodplants of *P. icarus*, were collected from the pollen and nectar margins of each field. Two composite samples per plant species were taken at each of the 10 equidistant sampling points along the margin of the two fields. The composite sample was taken from a 1 m radius circle at 10 points along the margin. For field A (cropped area = 7.28 ha) the centre of the sample circles were 35.5 m apart, with a sample point at each end (margin of 320 m). For field B they were 28 m apart (margin length 250 m, cropped area= 15.71 ha).

Two grams of leaves were taken from all parts of multiple plants and were stored in plastic bags lined with aluminium foil at -80°C until time for sample preparation. One gram of sample foliage was pulverised with a mortar and pestle in liquid nitrogen and analysed for the presence of neonicotinoids (clothianidin, thiamethoxam, imidacloprid, acetamiprid and thiacloprid) with ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) (following methods described in detail in Botías *et al*. (2016)). Samples were subsequently re-extracted from *L. corniculatus* and *T. hybridum* at the sites which had shown high levels of clothianidin in clover to confirm the high levels of clothianidin found in the *T. hybridum* samples and the low concentrations found in the *L. corniculatus* samples. Where these levels differed in the second extract, the lower results were used in the statistical analyses.

*Laboratory-based study*

*Experimental contamination of vegetation*

Individual 6cm pots of *Trifolium repens* (0.2 g of seed per pot) (Crocus, Surrey, England) were sown in multipurpose growing medium (50 g per pot) (B&Q, Brighton, United Kingdom, own brand) and grown under greenhouse conditions. *T. repens* was grown instead of the *T. hybridum* that had been sampled in the field due to space constraints (it is a much smaller plant). Pots were placed onto a deep plastic tray, through holes cut in a cardboard spacer which protected the watering solution from light exposure, to minimise UV-degradation of the pesticide.

Clothianidin (analytical grade; Sigma-Aldrich, Gillingham, UK) solutions of 0ppb (control), 5, 15, 50 and 500 ppb were made up on alternate days from a stock solution diluted with acetone stored in a -80 $^{o}$C freezer. Solutions were watered directly into the tray so that no solution touched the leaves. Each treatment group's tray was watered with 500ml each time. Plants were kept in the contamination tray for 2 weeks before the foliage was presented to the larvae. Seeds were continuously sown and new pots of *T. repens* were added to the contamination trays daily to ensure there was a continuous supply of contaminated foliage available that had been exposed for two weeks. Effective concentration levels in the leaves were analysed by taking a composite sample of foliar tissue from each treatment group two weeks after the initial dosing of the plants, and analysed using the same method that was used for the farm samples.

*Obtaining eggs*

Female *P. icarus* were captured at two different sites in Brighton, East Sussex (50.860343, -0.120088 and 50.869376, -0.085992) between the 7$^{th}$ and the 10$^{th}$ June 2016. These individuals were kept in 6L plastic aquariums in a temperature-controlled laboratory (23$^{o}$C). Sponges soaked in a mixture of orange sports drink (Asda own brand, Asda, UK), simple syrup (a thick sugar liquid made from equal measures of water and caster sugar) and soy sauce (Kikkoman) (approximately 14: 1: 0.05) were provided for a food source. *L. corniculatus* seeds were sown in 12cm pots with a multipurpose growing medium (B&Q, Brighton, United Kingdom, own brand), and were provided as oviposition substrate when approximately 4 weeks old.

The plants were replaced every three days and eggs were counted. These plants were put into a holding aquarium with the same conditions and temperature. Once ~200 eggs had been laid

within a 2-day period, these plants were placed in a climate control chamber at 24°C, with 70% humidity to stimulate egg hatching. Eggs were obtained from 34 females in total.

*Exposure of larvae*

Seven day old larvae (from date of hatching) were randomly assigned across the five treatment groups into individual 9 cm petri dishes lined with moistened filter paper; half of the lid was covered with black tape to provide shading. These were kept in a growth chamber at 70% humidity, 25°C and a 16:8 hr day : night cycle (Goverde *et al*., 1999). There were 30 replicate larvae per group. Larvae were fed *ad libitum* with leaves of *T. repens* sampled from plants that had been in the contamination tray for exactly two weeks. The stems of the leaves were inserted into 1.5ml Eppendorf tubes with a pierced cap filled with mineral water (Asda own brand, Asda, UK) to prevent the leaves from wilting (Goverde *et al*., 1999), and leaves were replaced every three days. After larval measurements had been taken (see below), using a soft paintbrush the larvae were carefully placed back into the petri dish, on fresh moistened filter paper.

*Monitoring development of larvae*

Measurements were taken every three days for a total period of 57 days. Larval length and width were measured to the nearest 0.01 mm using a microscope with graticule. Time to pupation was also recorded, and death was determined either when the larvae failed to respond when gently touched with the paintbrush or when there was cessation of food consumption for 5 days or more. The development time to specific instars could not be monitored accurately due to the resting habit of Lycaenidae larvae tucking their head capsule underneath their bodies, which meant that head capsule width could not be recorded without disturbing the individual.

As soon as the first larva had pupated, larvae were checked twice a day (09:00 and 18:00) to record pupation date. Pupae were left for 24 hours after pupation so that they could harden, before being weighed on a 0.001 g resolution balance (Precisa 125A, Newport Pagnell, Bucks, UK) and then were placed in clear plastic cups lined with dampened paper towel inside the growth chamber. They were checked twice a day (09:00 and 18:00) for emergence. Once emerged, adults were left in situ for either 9 or 15 hours (until the next check time) in order to allow for their wings to fully unfurl. They were then killed by placing them back in to their plastic cups and into a -80°C freezer. Once dead, they were weighed on a 0.001g resolution balance.

*Data Analysis*

All analyses were performed in SPSS (IBM SPSS Inc., v. 22, USA).

*Environmental samples*

A Mann-Whitney U test was used to determine if there were differences in clothianidin and thiamethoxam concentrations between *T. hybridum* and *L. corniculatus*, and between the experimental fields. Because of dissimilar distribution shapes, rank means of contamination levels in foliage are reported. To perform the statistical analyses, all concentrations that were over the limits of detection (≥MDL) but below the limits of quantification (<MQL) were assigned the value considered as the MDL in each case (Table 5.1). Concentrations below the MDL were considered to be zero (Botías *et al*., 2016).

**Table 5.1** Numbers of samples analysed, frequencies of detection and means (± standard deviation) of neonicotinoid levels found in vegetation samples collected from *T. hybridum* and *L. corniculatus* growing in a pollen and nectar margin. Details of the planting history of each field can be found in Figure 5.1. MDL is the method detection limit, MQL is the method quantification limits.

| **Origin of samples** | **N** | | **Thiamethoxam** MDL = 0.1 (ng/g) MQL = 0.3 (ng/g) | **Clothianidin** MDL = 0.2 (ng/g) MQL = 0.6 (ng/g) |
|---|---|---|---|---|
| **Pollen and nectar margin in Field A -** Time from clothianidin introduction to sampling: **10 months** | | | | |
| *Lotus corniculatus* | 10 | Frequency of detection (%) | 60% | 10% |
| | | Range (ng/g) | 0 - <0.10 | 0- <0.20 |
| | | Mean ± SD (ng/g) | 0.06 ± 0.05 | 0.02 ± 0.06 |
| *Trifolium hybridum* | 10 | Frequency of detection (%) | 90% | 80% |
| | | Range (ng/g) | 0 – 0.54 | 0 – 48 |
| | | Mean ± SD (ng/g) | 0.30 ± 0.16 | 6.31 ± 14.17 |
| **Pollen and nectar margin in Field B -** Time from clothianidin introduction to sampling: **21 months** | | | | |
| *Lotus corniculatus* | 10 | Frequency of detection (%) | 10% | 80% |
| | | Range (ng/g) | 0 - <0.10 | 0 – 0.91 |
| | | Mean ± SD (ng/g) | 0.01 ± 0.03 | 0.30 ± 0.31 |
| *Trifolium hybridum* | 10 | Frequency of detection (%) | 0% | 80% |
| | | Range (ng/g) | n/a | 0 - 41 |
| | | Mean ± SD (ng/g) | n/a | 12 ± 15.75 |

*Laboratory study*

Calculations investigating the impact of clothianidin on larval size were performed on a subset of data which excluded time points at which pupation had started to occur. Larval size ($mm^2$) across treatment groups was compared using repeated measures ANOVA with the treatment-by-time interaction as the primary effect of interest. Data were log-transformed in order to meet the assumptions of normality (as tested by the Shapiro-Wilk statistic and visual inspection of Q-Q plots) and also tested for homogeneity of variances prior to analysis. The assumption of sphericity (as defined by Mauchly's statistic) was not met for data from any treatment group, therefore Greenhouse-Geisser adjustments were made to correct the ANOVA and it is this adjusted p-value that is reported. Further pairwise comparisons were examined using Dunn's procedure with a Bonferroni correction for multiple comparisons.

For analysis of larval survival, all larvae that reached the pupal stage were defined as survivors, irrespective of whether they later successfully completed metamorphosis (Haider *et al.,* 2013). Survival of the larvae across the treatment groups was analysed using Kaplan-Meier survival analysis, and the log-rank test with a Bonferroni correction was applied to test for differences between patterns of survival distributions. Censored data (i.e. the number of larvae reaching pupation) across treatment groups were dissimilar and are therefore reported (Table 5.2). Four larvae were still alive at the end of the experiment (11$^{th}$ August) (15 ppb n=1, 50 ppb n=3) and were excluded from the survival analysis.

Generalized Liner Models (GLM) were used to analyse pupal stage duration, adult weight, pupal weight, and time taken to reach pupation with treatment and sex as the predicting factors. Normal error distribution was stipulated for pupal weight, adult weight analysis, pupal stage duration and time taken to reach pupation. We first fitted full models and systematically omitted interaction terms if they did not increase model fit. Models were fitted to the data using Akaike Information Criterion (AIC).

## 5.4 Results

*Environmental samples - Vegetation contamination and residue analysis*

*Clothianidin*

Overall clothianidin residues in field margin plants ranged from 0 – 48 ng/g (Table 5.1). Levels found in *L. corniculatus* were higher in field B, where time between sowing and sampling was 21 months, than those in field A where time between sampling and sowing was less at 10

months (Mann Whitney U test: U= 86, z= 3.058, p= 0.005; mean rank: field A 6.9, field B 14.1, mean ± SD: field B 0.30 ± 0.31 ng/g, field A 0.02 ± 0.06 ng/g). Levels of clothianidin in field A were much higher in *T. hybridum* than those found in *L. corniculatus* (6.31 ng/g ± 14.17, 0.02 ng/g ± 0.06, respectively (Table 5.1); Mann Whitney U test: U= 86.5, z= 3.069, p= 0.004; mean rank: *T. hybridum* 14.15, *L. corniculatus* 6.85). There was no statistical difference in clothianidin levels between species in field B (Mann Whitney U test, U= 67, z=1.353, p= 0.218). There was no statistical difference in clothianidin levels in *T. hybridum* between fields A and B (Mann Whitney U test: U=58.5, z= 0.667, p= 0.529). Frequency of clothianidin detection was also higher in field B (*L. corniculatus* 80%, *T. hybridum* 80%) compared to field A (*L. corniculatus* 10%, *T. hybridum* 80%).

*Thiamethoxam*

Foliar thiamethoxam residues ranged from 0 to 0.54 ng/g in field A which had the shortest time between sowing and sampling. In field B, thiamethoxam was only detected in *L. corniculatus* (0.01 ± 0.03 ng/g, frequency of detection: 10%) with levels in *T. hybridum* being lower than the method detection limit (MDL; ≤0.1 ng/g)). Frequency of thiamethoxam detection was higher in *T. hybridum* (Field A: frequency of detection in *L.* corniculatus 60% and *T. hybridum* 90%).

If the results from both margins are combined to provide a more general field-realistic contamination overview irrespective of the time when the treated seed was planted, the mean clothianidin levels found in *T. hybridum* were 9.04 ng/g ± 15.22 (n=20), and 0.16 ng/g ± 0.26 in *L. corniculatus* (mean ± SD). Imidacloprid, acetamiprid and thiacloprid were not detected in the samples.

*Larval survival*

We found that our laboratory based *T. repens* leaf contamination method produced very similar concentrations in the foliage to those that were in the solutions (Mean result, n=2 of each treatment group: Control =0 ng/g; 5 ppb = 5 ng/g; 15 ppb =14.5 ng/g; 50 ppb= 53.9 ng/g; 500 ppb = 439.1 ng/g).

Individuals in the 500ppb group experienced higher levels of mortality than the lower, field-realistic treatment groups (Kaplan-Meier analysis, log rank: $\chi^2(4)=11.211$, p= 0.024 (Figure 5.2)), with a clear drop in survival at the start of the experiment (day 9). Post-hoc pairwise comparisons (Kaplan-Meier analysis, pairwise log-rank tests) highlighted significant differences

between the control and 500ppb ($\chi^2(1)= 5.337$, p= 0.021) and 15ppb and 500ppb ($\chi^2(1)=5.337$, p= 0.018. Percentage survival was lowest in the 50ppb treatment group (63%) with all the treatment groups bar the control being in a tight range (63% – 73.3%). The greatest survival was in the control group (90%) (Table 5.2).

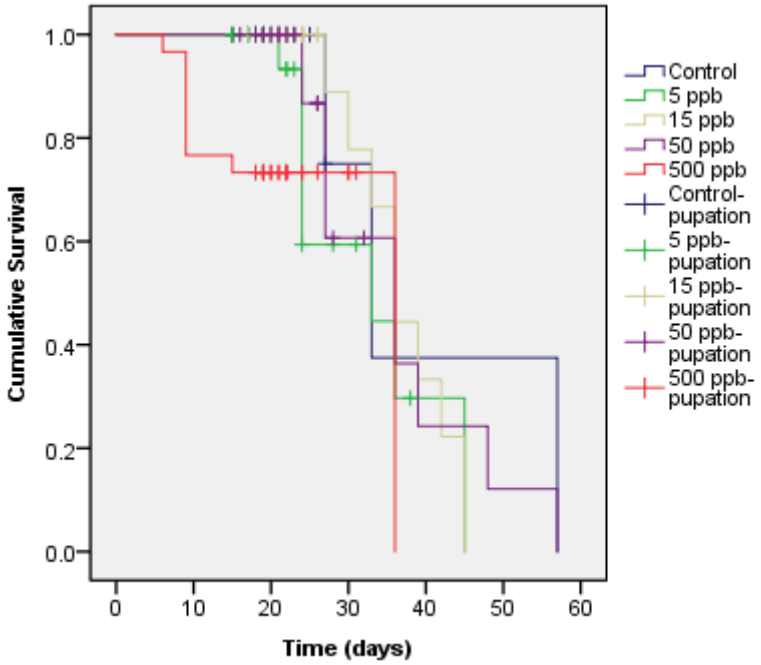



**Figure 5.2** Cumulative larval survival across five treatment groups. Post-hoc pairwise comparisons (Kaplan-Meier analysis, pairwise log-rank tests) showed significant differences between the control and 500 ppb ($X^2(1)= 5.337$, p= 0.021) and the 15 ppb and 500 ppb ($X^2(1)= 5.597$, p= 0.018). Crosses indicate individuals that reached the pupal stage.

*Larval development*

Three larvae in the 50 ppb group and one individual in the 15 ppb group were still alive at the end of the experiment; one control replicate escaped at the beginning of the experiment, and the rest of the larvae had either died or pupated. These larvae still responded to light touch but had not consumed any food for 5 days. After five days of not feeding, these larvae were removed from the experiment and freeze-killed.

Clothianidin had a significant effect on larval size (Figure 5.3, RM ANOVA, $F_{4, 126}$=3.632, p= 0.008) with a significant pairwise difference between the control and 15 ppb (Dunn's with Bonferroni correction, p=0.024), indicating that 15 ppb is the minimum growth inhibition concentration. The growth rate of larvae in the control, 5 ppb, 15 ppb and 50 ppb all followed similar patterns of growth over time with the control group exhibiting the fastest initial growth rate; the growth of individuals in the 500 ppb treatment group was delayed in the initial stages but appears to exhibit a faster growth rate from day 9.

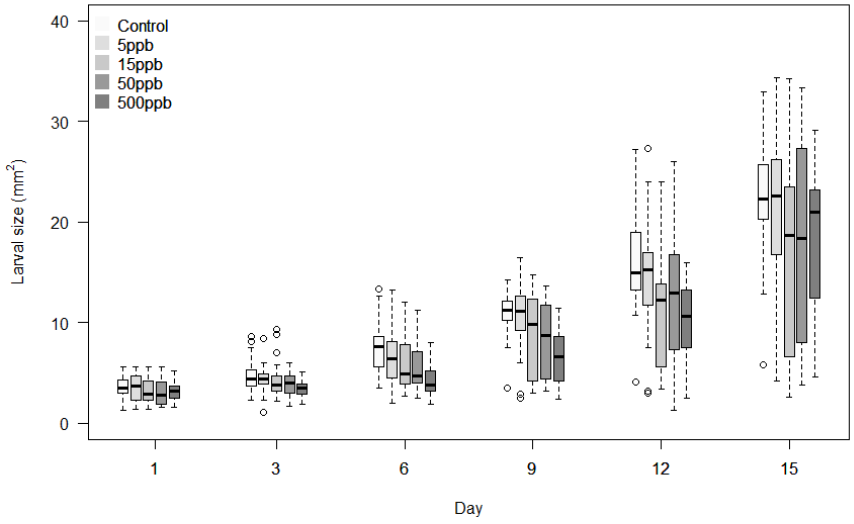



**Figure 5.3** Average area ($mm^2$) of *P. icarus* larvae. Analysis was performed on a subset of data which excluded time points at which pupation had started to occur. All larvae were included in investigation of size, regardless of whether they survived to pupation.

There was no significant effect of treatment on development time (start of the experiment to the pupal stage) ($F_{4,91}$= 0.306, p=0.860, GLM), pupal weight ($F_{4, 91}$ = 0.797, p= 0.530, GLM), adult weight ($F_{5,90}$=0.394, p=0.852) or the duration of the pupal stage ($F_{5,90}$ = 1.759, p=0.140, GLM). The highest levels of pupation were observed in the control group (26 individuals) with the lowest observed in 50 ppb (17 individuals) (Table 5.2). Median time to pupation in the control and 5 ppb groups was 3 days shorter (33 ± 4.58; 33 ± 10.1) than those in the 15 ppb, 50 ppb and 500 ppb groups (36 ± 3.16; 36 ± 6.48; 36) (Table 5.2).

**Table 5.2** Larval survival, development time and observations from five different larval populations reared on vegetation contaminated with clothianidin. Treatments sharing the same letter (A, B) did not differ significantly at P < 0.05 (post hoc test: pairwise log-rank test using Bonferroni correction). The post-hoc comparisons test the entire curve of mortality for each treatment group, and so the earlier mortality levels in the 500 ppb group are likely to be the cause of the significant difference in survival distributions. [a]Due to the survival function not reaching .45, standard errors and 95% confidence intervals could not be calculated.

| Treatment Group | Number of larvae that reach pupation (total n of group) | Survival (%) | Days to pupation | | Pupal weight (g) |
|---|---|---|---|---|---|
| | | | Median (± SE) | Range | |
| Control (**B**) | 26 (29) | 89.7 | 33 ± 4.58 | 24.02 - 41.98 | 0.0324 |
| 5ppb (**AB**) | 22 (30) | 73.3 | 33 ± 10.1 | 13.20 – 52.8 | 0.0363 |
| 15ppb (**B**) | 20 (29) | 69 | 36 ± 3.16 | 29.8 – 42.2 | 0.0337 |
| 50ppb (**AB**) | 17 (27) | 63 | 36 ± 6.48 | 26.29 – 51.7 | 0.0328 |
| 500ppb (**A**) | 21 (30) | 70 | 36 | [a] | 0.0343 |

## 5.5 Discussion

This study gives a first estimate of the levels of neonicotinoid contamination in margin vegetation grown specifically to be attractive to pollinators. In the field, clothianidin was found in both *L. corniculatus* and *T. hybridum,* but contamination levels varied markedly between the two species, with much higher levels of clothianidin detected in *T. hybridum.* The maximum concentration of clothianidin we found in *T. hybridum* (48 ppb) is higher than that which has been found in previous studies of the contamination of non-target vegetation (Rundlöf *et al*. (2015): field border plants, ≤ 2 days after sowing of treated crops: 1.2 ± 0.8ppb, ≤ 2 weeks after sowing: 1.0 ± 0.8ppb; Pecenka and Lundgren, (2015): milkweed, 1.14 ± 0.10 ppb clothianidin, maximum of 4 ppb). Sampling occurred at the later stages of the margin's seasonal lifespan (a week before the margin was to be mown), and 10 or 21 months after neonicotinoids were deployed, so it is highly likely that clothianidin levels would have been higher had we sampled earlier.

Our results suggest that the contamination of vegetation by clothianidin may be more likely when wildflowers are planted directly into soil that has previously supported a crop grown from neonicotinoid treated seed, compared to contamination levels of a flower margin grown alongside a treated crop. However, it is important to note that in this respect we did not have true replicates of each field margin, and so differences between pesticide levels in the two margins may be due to other unknown factors. Nonetheless, our results provide a proof-of-concept; sowing wildflowers either adjacent to or onto contaminated soil results in contaminated plants.

The differences in levels of contamination between *T. hybridum* compared to *L. corniculatus* are likely to be due to a difference in plant physiology, but this has never been studied and would be valuable to investigate further. Vegetation sampled from plants 10 months after sowing with Redigo Deter© treated wheat (Field A) seed frequently also contained thiamethoxam. With no history of thiamethoxam use on the farm, it is possible this compound has been introduced to the soil via dust drift or soil dust from a neighbouring farm (Limay-Rios *et al.,* 2016), from contaminated machinery or seeds, or from contaminated soil water from a neighbouring farm. Similarly, Botías *et al*. (2015) found thiacloprid in wildflower samples from several farms with no history of its use.

Our data suggest that neonicotinoid levels used in the experimental chronic exposure of the larvae in this laboratory-based investigation are a true reflection of likely field exposure, apart from that of the positive control (500 ppb). A lethal effect of clothianidin on *P. icarus* larvae was evident, but there was no linear relationship between dose and mortality. The only treatment that had a significant effect on early mortality was 500 ppb, which is ten times that of the highest field observed concentration. Across a range of insects tested, previous risk assessments have shown that for clothianidin and other neonicotinoids, $LD_{50}$'s for non-targets range from 3.7 ppb to as much as 81 ppb (Pecenka and Lundgren, 2015). We are unable to calculate an $LD_{50}$ value since even at the highest dose used more than 50% of larvae survived, suggesting that our study species is less susceptible to this chemical than other species tested so far. However, we did find that clothianidin negatively affected early stage development of the larvae, with larval size being reduced by the presence of field realistic levels. Larvae of *D. plexippus* (Nymphalidea) take longer to develop, with first instars having reduced body weight and reduced length when exposed to clothianidin in their diet; this effect was not detected in second instars (Pecenka and Lundgren, 2015). This variation in the sensitivity of larval stages has also been observed in *Cydia pomonella* (Tortricidae), with first instar caterpillars being more than 100 times more sensitive than fifth instar caterpillars (Stará and Kocourek, 2007). The contamination of larval food sources has a detrimental effect on early stage development and our data add to growing evidence that the pollution of non-target vegetation may be detrimental to early herbivorous larval stages. Overall, clothianidin had no effect on time to pupation due to apparent compensatory growth in the later stages. Costs for compensatory, accelerated growth periods include increased rates of mortality and decreased longevity (Mangel and Munch, 2005). Further work is required to investigate if this compensatory growth phase had a negative impact on the resulting adult as lower fecundity is associated with decreased pupal development time in some Dipteran species (Telles-Romero *et al.,* 2011).

As well as considerable variation in the sensitivities of species to neonicotinoids, and the different toxicity of the neonicotinoid compounds, there are also many documented studies showing neonicotinoid resistance in ecologically relevant species (Pisa *et al.,* 2015). Encouragingly, the survival of some larvae in the 500ppb group to adulthood is a strong indication that there exists a physiological ability to cope with the toxin at the larval stage.

The presence of neonicotinoids in marginal vegetation is a cause for concern, as these levels can overlap with $LC_{50}$ values for non-target, important insects (Botías *et al.,* 2016). There is a pressing need to mitigate against non-target contamination by neonicotinoids in the field,

especially since these margins are grown to boost pollinator populations, to attract natural enemies of arthropod pests (Botías *et al.,* 2015) and to provide a food source for herbivorous species and larvae.

# **Chapter 6** - Multivariate analysis of the change in bird abundance and arable agricultural practice in England: population decline is associated with potential ecological exposure to neonicotinoid use

Chapter 6 is written in the style of an article appropriate for PeerJ

Basley, K.; Shaw, P.;Gibbons, D.; Wilson, J.; Goulson, D. Multivariate analysis of the change in bird abundance and arable agricultural practice in England: population decline is associated with potential ecological exposure to neonicotinoid use.

DGo, JW, and DGi and KB were responsible for the study design. KB collected the data. KB, PS, DGo and JW analysed the data. KB, DGo and JW wrote the manuscript

## 6.1 Abstract

Agricultural change is a dominant driver of biodiversity change and the major current, and likely future, threat to European wildlife. In the UK, many farmland bird species have undergone significant declines in recent decades. Ordination techniques were used to identify patterns of change in both agricultural variables and farmland bird species that occurred in England from 1996-2013, broadly following the approach of Chamberlain *et al*. (2000) who focused on change during 1962-1995.

We show that patterns of change in bird abundance and agricultural variables are broadly correlated. Overall change in farming practices has not been steady, but instead showed periods of stability (1996-97, 1998-2000 and 2008-12), with rapid shifts between 2000-02 and 2012-13 and a yearly change from 2004 to 2007. These rapid shifts can be plausibly linked to foot and mouth disease, the phasing out of set-aside (the policy of taking land out of production to reduce crop surpluses) and consequences of the Common Agricultural Policy reform, respectively. Changes in farmland bird populations over time were more even, and did not coincide directly with periods of rapid agricultural change. A rapid decrease in bird populations occurred between 1998-2000 and a further sustained decrease was observed from 2005-2009, which are likely to be the result of lagged response to changes in farming that occurred a year or two earlier. Populations of those bird species included in these analyses were relatively stable in the most recent years examined (2010-13). For each species, we used a trait-based approach to estimate their likely exposure risk to the direct and indirect adverse impacts of neonicotinoid insecticides, and found that species with the greatest exposure risk had declined the most. This work is further evidence that monitoring the impacts of neonicotinoids in our environment is urgently required, and that forming links between agricultural and bird population change is an important approach that will aid the conservation of bird species in agricultural landscapes.

## 6.2 Introduction

Agriculture is a globally dominant driver of biodiversity change (Tilman & Lehman. 2001). The clearance of land for crops and the intensive management strategies used to increase production, also reduce the value of farmed land for wildlife (Green *et al*. 2005). The European Commission's mid-term review of the 2020 biodiversity strategy has shown that EU member states are still failing to protect wildlife species and important habitats, with no measurable

improvement in the status of the majority of species typically associated with agricultural environments since the last reporting period in 2010 (European Commission 2015). As it stands, agricultural management and climatic change are the perceived major drivers of biodiversity change in the UK (Burns *et al*., 2016). Birds have been adopted as a particular focus for biodiversity conservation (Butler *et al.* 2007*)*, as their populations are sensitive indicators for the effects of agricultural intensification (Butchart *et al*. 2010, Wilson *et al*. 2010), and are commonly used as a proxy for wider biodiversity health on farmland (Butler *et al*. 2010). The UK is one of the countries in which the impacts of agricultural intensification on bird populations has been most severe (Donald *et al*. 2001) and, due to the efforts of the many volunteers working alongside professional organisations, UK birds are one of the best-monitored taxonomic groups in the world (Eaton *et al*. 2015), making them especially good indicators of the impacts of land use change.

Fuller *et al*. (1995) identified four interrelated trends in UK agricultural practices with important implications for wild bird populations, which have subsequently been investigated further by others. Firstly, the replacement of spring sowing of cereals by autumn sowing has resulted in a dramatic reduction in the availability of over-winter crop stubbles, formerly an important foraging resource for seed-eating birds (Butler *et al*. 2010). Secondly, the simplification of crop rotations to yield landscapes dominated by either arable crops or grassland has increased the uniformity of the countryside and reduced food and nest site availability for birds (e.g. Benton *et al*. 2002). Thirdly, increases in fertiliser and pesticide usage have caused declines in the invertebrate and weed seed foods of many farmland birds and is known to have caused the declines of some species (e.g. Kuipjer *et al*. 2009). Lastly, grassland management has also intensified such that grassland is re-sown with more competitive species, drained, fertilised, and subjected to higher grazing pressures, and forage grasses are cut earlier and more frequently. This has resulted in grassland with greatly reduced wildlife interest, with reduced nesting and foraging opportunities for birds (Vickery *et al.* 2008).

Chamberlain *et al.*'s (2000) ordination study of multivariate change in agricultural practice and multi-species change of farmland bird populations in England and Wales between 1962 and 1996 confirmed that the two trends were broadly matched, but with bird population change tending to lag agricultural change, and concluded that large shifts in agricultural management were a plausible cause of farmland bird decline. Subsequent studies have established both the mechanisms linking agricultural intensification and decline, and those linking agri-environment

interventions and population recovery for many species of bird (e.g. Newton 2004, 2017, Wilson *et al*. 2009, Wilson & Bradbury 2015).

Although greening measures have been progressively incorporated into the EU's Common Agricultural Policy (CAP) to improve environmental sustainability (Baldock *et al*. 1996), the impact of these on biodiversity recovery has been limited (Kleijn & Sutherland 2003; Kleijn *et al.* 2011, Pe'er *et al*. 2014), and novel aspects of agricultural intensification continue to emerge. Notable examples include, the growing use and potential environmental impact of veterinary pharmaceuticals in agricultural systems (Margalida *et al*. 2014), and the development and adoption of new classes of pesticide, notably neonicotinoids which are now the most widely used group of pesticides in the world (Jeschke *et al*. 2011). The widespread use of neonicotinoids, together with their systemic properties in the plant, and water solubility, mean that many non-target organisms in agricultural landscapes are likely to be exposed (Goulson, 2013). Research in the Netherlands found that declines in insectivorous birds were associated with high neonicotinoid surface water contamination, suggesting neonicotinoids may pose a threat to birds by causing a depletion in food resources (Hallmann *et al.* 2014). In experimental studies, exposure to imidacloprid had reproductive effects including reduced clutch size, delayed laying dates and depressed offspring immune response in red-legged partridges (*Alectoris rufa*) (Lopez- Antia *et al*., 2016), and clothianidin exposure reduced germ cell numbers in Japanese quail (*Coturnix japonica*) (Hoshi *et al*., 2014). However, despite this initial evidence for the negative impacts of neonicotinoids on birds, population level effects remain poorly studied. (Gibbons *et al* 2015; Pisa *et al*. 2017).

In this study, we first update the study by Chamberlain *et al*. (2000) by testing the relationship between multivariate changes in English agricultural variables (Table 6.1) and multi-species change in bird populations on farmland in England for the period 1996-2013. Secondly because neonicotinoid pesticides are a new, but rapidly increasing component of arable agricultural practice in England since the mid-1990s, we also test whether there is any association between variation in bird species population trends and variation in likely exposure of different species to any direct or indirect impacts of neonicotinoid use, based on species' ecological traits.

## 6.3 Methods

*Agricultural data*

Agricultural data were derived from three statistical datasets maintained by the UK Department of Environment, Food and Rural Affairs, covering crop area (ha x $10^3$) DEFRA (2015a), the British survey of fertiliser usage (kg $ha^{-1}$) (DEFRA 2015b), and DEFRA's pesticide usage statistics website (measured as kg of active ingredient applied) (DEFRA 2015c). Only variables with complete runs of data covering all years were used. There are no data for fertiliser application in England alone and so this is reported as a combined variable for England and Wales. Further details on the derivation of data from these sources can be found in Table 6.1. Pesticide input data are only available every second year, and so the mean of two survey years was used to estimate usage in the intervening year, following Chamberlain *et al*. (2000). Agricultural variable data were smoothed in SPSS version 22 using the 4235-H twice running median smoothing technique so that underlying trends could be observed (Chamberlain *et al*. 2000). These smoothed trends are presented in Figure S6.1. Data were standardised with each point returning a normalized value characterised by its mean and standard deviation. Unsmoothed, standardised agricultural data were used in all analyses.

**Table 6.1** Agricultural variables included in PCA analysis.

| Variable | Years available | Source | Unit of Measurement |
|---|---|---|---|
| Barley (autumn sown) (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Barley (spring-sown) (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Fertiliser application (N+P+K on all crops and grass) | 1996 – 2013 | DEFRA, 2015b | Kg/ha |
| Permanent grass (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| New grass (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Linseed (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Oats (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Oilseed rape (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Rough grazing (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Set-aside (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Potato (total, early, main) (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Sugar beet (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Total tilled land (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Wheat (total) (area) | 1996 – 2013 | DEFRA, 2015a | Thousand ha |
| Fungicides (total) | 1996 – 2013 | DEFRA, 2013 | Spray area (ha) |
| Herbicides (total) | 1996 – 2013 | DEFRA, 2013 | Spray area (ha) |
| Insecticides (total) | 1996 – 2013 | DEFRA, 2013 | Spray area (ha) |
| Neonicotinoids (sum of applied imidacloprid, thiamethoxam and clothianidin) | 1996 – 2013 | DEFRA, 2013 | Total weight (kg) |

*Bird population data*

Data were obtained from the UK Breeding Bird Survey (BBS) for 33 species that are either largely dependent on, or are known to heavily use farmland. The BBS is based on randomly assigned Ordnance Survey 1km squares where the observer walks two 1km transects, twice, between April and June, with approximately 3700 squares surveyed annually in the UK, and 2800 of these in England (Harris *et al*. 2017). Full species lists, with their associated population

change and habitat niche are shown in Table 6.2. Species data for England were provided as a smoothed set of population indices for each species from 1996 – 2013 in the format of annual percentage change. Population trends had been smoothed using a thin-plate smoothing spline with degrees of freedom about one third of the total number of years (Robinson *et al*., 2016; Fig. S6.2a, b).

**Table 6.2** Bird species considered in PCA analysis. Population trend (1995 – 2013) has had end years truncated and data smoothed. F: Farmland, W: Woodland (y*et al*so commonly use farmland). S: specialist G: generalist (Gibbons *et al*., 1993, Fuller *et al*., 1995). Trends are England specific based on smoothed trend data. $^{+}$Indicates a significant change. *Barn owl data comes with the caveat that nocturnal species are poorly recorded through the Breeding Bird Survey. $^{\&}$Indicates an extra species added to analysis, not originally included in Chamberlain *et al*. (2000)

| **Bird Species (BTO code)** | **Population trend 1995-2013** | **Specialism** | **Amber listed** | **Red listed** |
|---|---|---|---|---|
| (BO) Barn Owl *Tyto alba**$^{+\&}$ | 219 | F / S | • | |
| (GO) Goldfinch *Carduelis carduelis*$^{+}$ | 106 | F / S | | |
| (SD) Stock Dove *Columba oenas* | 11 | F / S | • | |
| (TS) Tree Sparrow *Passer montanus*$^{+}$ | 77 | F / S | | • |
| (WH) Whitethroat *Sylvia communis*$^{+}$ | 36 | F / S | • | |
| (S.) Skylark *Alauda arvensis*$^{+}$ | - 23 | F / S | | • |
| (LI) Linnet *Linaria cannabina*$^{+}$ | - 24 | F / S | | • |
| (Y.) Yellowhammer *Emberiza citrinella*$^{+}$ | - 25 | F / S | | • |
| (L.) Lapwing *Vanellus vanellus*$^{+}$ | - 27 | F / S | | • |
| (CB) Corn Bunting *Emberiza calandra*$^{+}$ | - 36 | F / S | | • |
| (P.) Grey Partridge *Perdix perdix*$^{+}$ | - 55 | F / S | | • |
| (SG) Starling *Sturnis vulgaris*$^{+}$ | - 60 | F / S | | • |
| (TD) Turtle Dove *Streptopelia turtur*$^{+}$ | - 91 | F / S | | • |
| (JD) Jackdaw *Coloeus monedula*$^{+}$ | 60 | F / G | | |
| (WP) Wood Pigeon *Columba palumbus*$^{+\&}$ | 41 | F / G | | |
| (RB) Reed Bunting *Emberiza schoeniclus*$^{+}$ | 28 | F / G | • | |
| (HY) Hobby *Falco Subbuteo*$^{\&}$ | - 7 | F / G | | |
| (RO) Rook *Corvus frugilegus*$^{+}$ | - 13 | F / G | | |
| (K.) Kestrel *Falco tinnunculus*$^{+}$ | - 27 | F / G | • | |
| (GR) Greenfinch *Chloris chloris*$^{+}$ | - 29 | F / G | | |
| (YW) Yellow Wagtail *Motacilla flava*$^{+}$ | - 40 | F / G | | • |
| (SF) Spotted Flycatcher *Muscicapa striata*$^{+\&}$ | - 61 | W / S | | • |
| (GT) Great Tit *Parus major*$^{+}$ | 30 | W / G | | |
| (B.) Blackbird *Turdus merula*$^{+}$ | 18 | W / G | | |
| (R.) Robin *Erithacus rubecula*$^{+}$ | 15 | W / G | | |
| (D.) Dunnock *Prunella modularis*$^{+}$ | 15 | W / G | • | |
| (ST) Song Thrush *Turdus philomelos*$^{+}$ | 10 | W / G | | • |
| (LT) Long-tailed Tit *Aegithalos caudatus* | 6 | W / G | | |
| (BF) Bullfinch *Pyrrhula pyrrhula* | 5 | W / G | • | |

| | | |
|---|---|---|
| (WR) Wren *Troglodytes troglodytes*[+] | 5 | W / G |
| (C.) Chaffinch *Fringilla coelebs*[+] | 5 | W / G |
| (BT) Blue Tit *Cyanistes caeruleus* | 2 | W / G |
| (LW) Lesser Whitethroat *Sylvia curruca* | - 1 | W / G |

*Data analysis*

A correlation matrix of the agricultural variables was produced to show similar or opposing trends in the data over time (Table S6.1). In R (R Core Team, 2013), Principal Components Analysis (PCA) was first used to reduce the dimensionality of the agricultural and bird abundance data, respectively, (Tables 6.3 and 6.4) and allow visualisation of relationships. The PCA of the bird data was dominated by the first principal component (BIRDPC1) which explained 65% of variation in the annual abundance indices (Table 6.3). The second principal component explained 23% of the variation in the annual abundance indices and distinguished those species with some evidence of higher abundance towards the middle of the times series ('peaked' trends), from others. The other principal components all had eigenvalues <1 and were not considered further. The first principal component of the agricultural variables (AGPC1) explained 54% of the variation in the data, and the second (AGPC2) explained a further 15% of variation with the remaining principal components all having eigenvalues <1. These four principal components were considered in detail in interpreting patterns of bird population and agricultural change. Greenfinch populations have been steadily declining due to trichomonosis. The stability of the model was explored by re-running it excluding greenfinch; this did not affect the results significantly.

Secondly, a trait database (Table S6.2) describing foraging ecology, diet and specialism to agricultural habitats was compiled for each of the species used in the analysis (excluding raptors) to quantify the extent to which each species' population may be exposed to neonicotinoid use in agricultural environments in England. Simple one or zero scores were used to minimise subjectivity and assessments were made by reference to Butler *et al.* (2007) and Robinson *et al*. (2016). Assessments draw on species' ecological requirements covering components of diet, foraging habitat and specialism (woodland or farmland). The summed score for each species is considered as a Neonicotinoid Exposure Risk (NER) score. The NER is based on the assumption that species feeding on invertebrates (both above and below ground), green crop material, and sown crop seeds will have a higher risk of exposure to neonicotinoids. Similarly, those that nest or feed in the crop will have a higher NER. The NER matrix was kept deliberately coarse, but further analyses would benefit from the inclusion of

other factors (i.e. weather). We then modelled the PCA loadings from BIRDPC1 (Table 6.3) against the summed NER score whilst controlling for migratory status (1 = migrant; 0 = non-migrant). Family was not included in the model as species came from approximately equally distant taxonomic groups, so phylogenetic correction could not be justified.

## 6.4 Results

*Bird population and agricultural change*

BIRDPC1 distinguished declining species (high values; Fig. S6.2a) from those with increasing, stable or fluctuating populations (lower values; Fig. S6.2b). The groups of consistently decreasing species (grey partridge, turtle dove, starling, rook, skylark, yellow wagtail, spotted flycatcher, corn bunting, linnet and yellowhammer) and consistently increasing species (woodpigeon, jackdaw, whitethroat, lesser whitethroat, tree sparrow, goldfinch, reed bunting, great tit and dunnock) are both ecologically and taxonomically diverse. Amongst the nine declining species, six had also been in decline during the preceding time period considered by Chamberlain *et al*. (2000) and only three (rook, yellow wagtail and yellowhammer) began to decline after 1996. Conversely, only two species - tree sparrow and reed bunting – showed increases during 1996 – 2013 after declines during the period considered by Chamberlain *et al*. (2000).

AGPC1 describes a trend over time from earlier years with more autumn barley, potatoes, sugar beet, stockfeed crops and fungicide use (the earlier years in the time series) to later years with more oilseed rape, permanent grass, spring cereals and herbicide and neonicotinoid use. AGPC2 distinguishes years with more set-aside and less tillage and wheat (before the end of compulsory set-aside policy in 2007; Gillings *et al*. 2010) from years with more tillage and less set-aside (after 2007). Figure S6.1 shows the trends in the key variables contributing to these two principal components.

*Relationship between agricultural and bird population patterns of change*

BIRDPC1 and AGPC1 are strongly correlated (Pearson r = -0.972, $P < 0.001$), as expected given that the two PCs each describe strong time trends in agricultural land use and practice and in bird populations. However, whilst the rate of change of BIRDPC1 is quite constant over 1996 – 2013, there is more variation in the rate of change of AGPC1, which demonstrates particularly sudden shifts between 2000 and 2002 and between 2012 and 2013 (Fig. 6.1). Moreover, in contrast with Chamberlain *et al*. (2000) the relationship between agricultural change and bird

population change is not clearly lagged with values of Spearman r for lagged relationships of varying length all lower (-0.920 – 0.966).

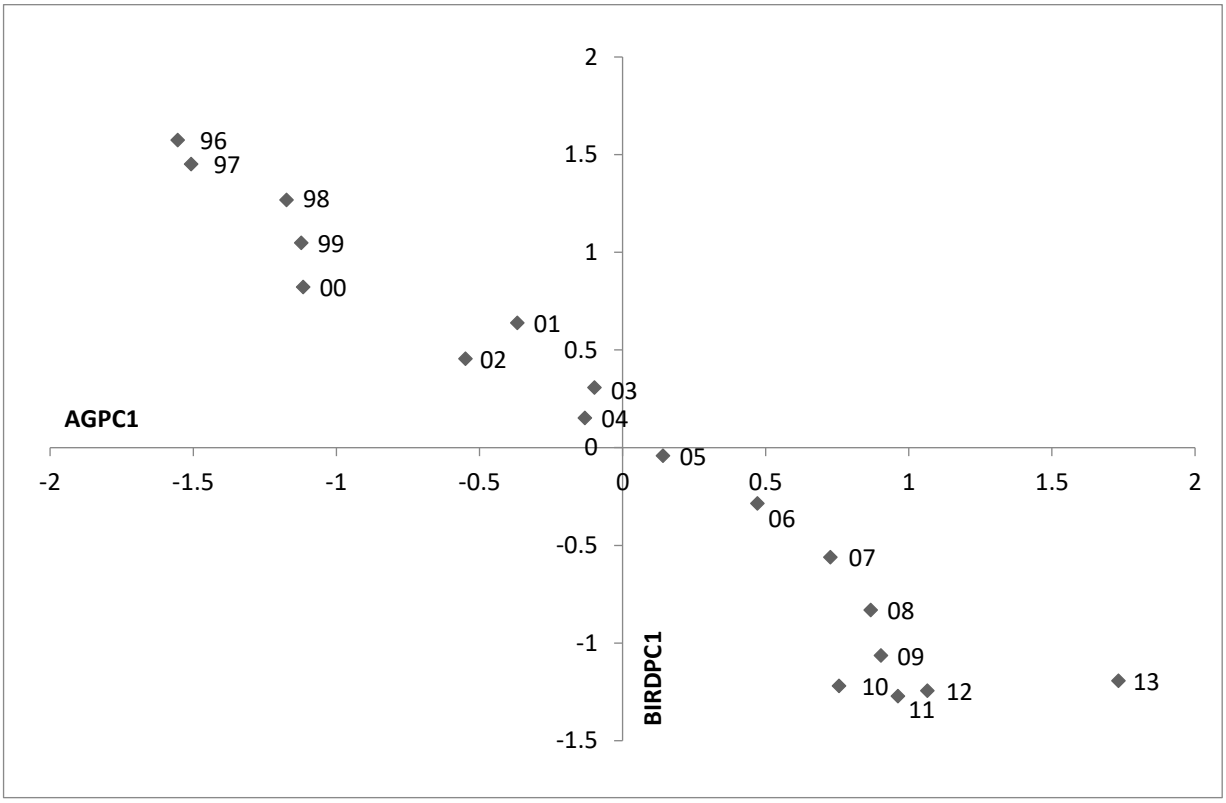



**Figure 6.1** AGPC1 (agricultural variables Table 6.1) plotted against BIRDPC1 (species from Table 6.2) (number plus diamond denotes a designated year from 1996 - 2013). AGPC1 represents 54% of the variation in all habitat data with a four-component solution. BIRDPC1 represents 65% of the variation.

BIRDPC2 and AGPC2 were also strongly correlated (Pearson r = -0.646, p = 0.004) with a small number of species – notably kestrel, lapwing, greenfinch, chaffinch and wren all showing changes in trend which broadly coincide with the ending of compulsory set-aside policy in the late 2000s. In all except the last two cases, this change in trend marks the start of a period of decline.

*Relationship between neonicotinoid exposure risk and population trend*

After controlling for the effects of migratory status, the loadings on BIRDPC1 were significantly associated with NER score (parameter estimate = 0.153 ± 0.055 SE, df = 27, t = 2.76, p = 0.0102), but there was no heterogeneity of slope of this relationship between migrant and non-migrant species. Thus, bird species are more likely to be in population decline if their NER score is high. Figure (6.2) plots the NER against the BIRDPC1 loadings, migrants are highlighted

in red. The species found in the lower left hand corner, with the higher NER scores are predominantly those species in decline (see Fig. S6.2a).

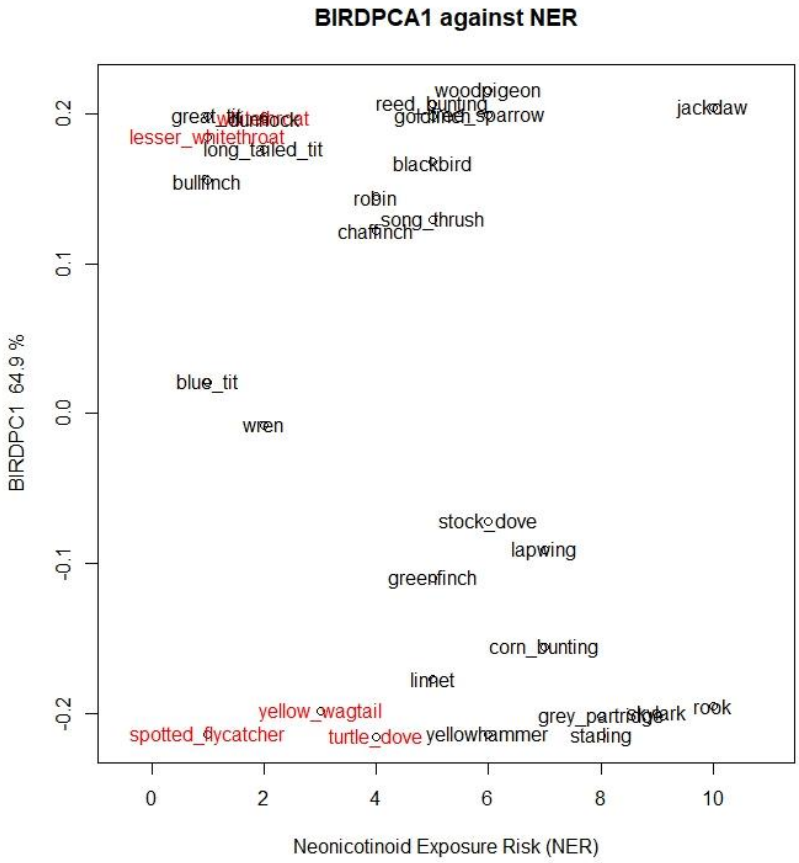



**Figure 6.2** Neonicotinoid Exposure Risk Score plotted against BIRDPC1. The species in the lower left hand corner with the higher NER score are predominantly those species in decline (See Fig S6.2a). Species in red are migratory.

## 6.5 Discussion

As between 1962 and 1995 (Chamberlain *et al*. 2000), we found that the first principal component of farmland bird population change distinguished increasing and declining species, and that the first principal component of multivariate agricultural change was strongly correlated with this variation in bird population trends. However, unlike the earlier study we found no clear evidence that bird population change lags agricultural change.

Grey partridge, turtle dove, starling, skylark, spotted flycatcher, linnet, yellowhammer and corn bunting all continue to decline but, since the mid-1990s, populations of rook, yellow wagtail and yellowhammer have shown declines that were not apparent during the earlier period of study. In contrast, long-term declines of song thrush, bullfinch, tree sparrow and reed bunting have halted or even been partially reversed, although in all these cases recovery is modest relative to the magnitude of the earlier decline (Robinson *et al*. 2016). Between 1962 and 1995, the greatest changes in agricultural practice were increases in the areas of oilseed rape and autumn cereals grown, and increased use of inorganic fertilisers and pesticides (Chamberlain *et al*. 2000). Since then, this study shows continuing increases in oilseed rape at the expense of other break crops, and in use of some pesticides, notably herbicides and, especially, rapid growth in use of neonicotinoid insecticides, primarily applied as seed treatments (Morrissey *et al*., 2015). This latter trend is consistent with the fact that neonicotinoids are widely used on both oilseed rape (Elbert *et al*. 2008) and cereals and have become the fastest-growing class of insecticides globally (Gibbons *et al*. 2015). In contrast, the trend towards replacement of spring-sown cereals with autumn-sown varieties has halted and been partially reversed, in part due to the increasing use of winter cultivation and spring sowing to assist in cultural control of herbicide-resistant black-grass *Alopecurus myosuroides* (Moss & Clarke 1994). A second axis of agricultural change marked the end of compulsory set-aside policy in 2007 and the return of most of this land area to arable production. However, onsets of population decline that were coincident with the loss of set-aside from the lowland farmland landscape in England were restricted to just three species, kestrel, lapwing, and greenfinch.

There is growing evidence that seed-eating birds which prey upon sown crop seeds could be exposed to dangerously toxic dosages of neonicotinoids if treated seeds are accessible at the soil surface (Lopez-Antia *et al.* 2016, Millot *et al*. 2017, Pisa *et al*. 2017). Evidence of indirect effects of neonicotinoid use on birds through reductions in invertebrate food supply remains scarce (Gibbons *et al*. 2015, Pisa *et al.* 2017), although Hallmann *et al*. (2014) found correlative evidence that insectivorous bird populations declined more at locations with higher surface water concentrations

of imidacloprid. In this context, and due to the significant association between population decline and NER score, the possible impacts of neonicotinoid usage should be considered for declining granivorous and insectivorous species including grey partridge, skylark, starling, yellowhammer and corn bunting. Of the species which showed correlated turning points in population trend at the same time as the landscape-scale withdrawal of set-aside, the onset of declines of kestrel, lapwing and greenfinch are all consistent with the fact that these species were amongst those found to be at higher density on set-aside than winter cereals both in summer and, in the cases of kestrel and greenfinch, in winter (Gillings *et al*. 2010). Kestrels may have benefitted from enhanced availability of small mammal prey on set-aside land (Macdonald *et al*. 2007), and there are studies both of set-aside and of agri-environment options designed to provide set-aside-like fallows as breeding habitat that nesting lapwings can benefit from these habitats (Watson & Rae 1997, Sheldon *et al*. 2004, 2007). In the case of greenfinch, whilst the loss of set-aside may have been influential, the emergence of trichomonosis as a novel, fatal disease of finches in the UK in 2005 is a more compelling coincidental cause of population decline (Robinson *et al*. 2010).

Our analysis also detected the ending of compulsory set-aide as a secondary, marked trend in agricultural practice over the period of interest. However, despite considerable concern that the loss of set-aside habitat might precipitate renewed declines of many farmland bird species benefitting from seed and invertebrate food resources and nesting habitat on set-aside (Gillings *et al*. 2010), there is relatively little evidence from this study that this is the case except, possibly for kestrel and lapwing. This may reflect the fact that the development and evidence-based improvement of agri-environment scheme measures has helped in part to compensate for the impacts of loss of set-aside (e.g. Sheldon *et al*. 2007).

Overall, a correlative study of this nature cannot prove the impact of specific agricultural changes on bird populations. However, causal links between agricultural change and bird population change have now been extensively studied across Europe and are both diverse and pervasive (Wilson *et al*. 2010). The continuing strong correlation found here between the first principal components of agricultural change and bird population change, as first found by Chamberlain *et al*. (2000), are consistent with their hypothesis that agricultural change continues to affect farmland bird populations. The fact that neonicotinoid use was the single most persistent and rapid recorded change in agricultural practice over the period 1996 – 2013 and that declining species tended to be those with higher NER scores suggest that more detailed studies of the potential direct, toxic impacts of neonicotinoids on granivorous species and the potential indirect effects via reduction in

invertebrate food supply on insectivorous species are worthy of more detailed study. This recommendation is further strengthened when our results are compared with those of Hallmann *et al*. (2014) who found significant inverse relationships between surface-water imidacloprid concentrations and population trend for eight (six after Bonferroni correction) of 15 species studied in the Netherlands. None showed a significant positive association. Five of these eight species were also considered by our study and four of these five (skylark, starling, yellow wagtail, and yellowhammer) were amongst those declining strongly, with whitethroat the exception.

## Appendix 6A – Supplementary Tables and Figures

**Table 6.3** Component matrix of bird species used in the PCA. Positive associations are with species with an increase in abundance, negative associations with BIRDPC are species that are declining. Four components extracted. Percentage variation explained; PC1: 64.9, PC2: 22.6, PC3: 6.5, PC4: 3.6

| | Component | | | | Communalities |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | |
| Grey Partridge | .933 | -.070 | -.307 | -.154 | .933 |
| Kestrel | .462 | -.863 | -.163 | -.107 | .996 |
| Hobby | -.829 | .357 | -.356 | .058 | .946 |
| Lapwing | .422 | -.899 | -.071 | -.060 | .995 |
| Stock Dove | .334 | .420 | .806 | -.152 | .962 |
| Wood Pigeon | -.996 | -.046 | -.005 | -.000 | .995 |
| Turtle Dove | .996 | -.016 | -.058 | .034 | .997 |
| Barn Owl | -.901 | -.377 | -.206 | .012 | .997 |
| Jackdaw | -.945 | .299 | .092 | -.016 | .992 |
| Rook | .902 | -.234 | -.168 | .247 | .958 |
| Skylark | .926 | -.217 | -.256 | -.149 | .993 |
| Whitethroat | -.917 | .342 | .116 | -.102 | .981 |
| Starling | .993 | -.022 | -.076 | .076 | .999 |
| Tree Sparrow | -.921 | .363 | .078 | -.115 | .999 |
| Yellow Wagtail | .918 | .352 | .011 | -.150 | .990 |
| Greenfinch | .508 | -.847 | .154 | -.013 | .999 |
| Goldfinch | -.916 | .384 | .036 | -.105 | .999 |
| Linnet | .819 | .318 | .284 | -.236 | .908 |
| Yellowhammer | .990 | .016 | -.007 | .016 | .981 |
| Reed Bunting | -.955 | -.128 | -.185 | -.163 | .988 |
| Corn Bunting | .720 | -.087 | -.632 | -.243 | .984 |
| Chaffinch | -.565 | -.812 | .101 | -.099 | .999 |
| Blue Tit | -.099 | -.608 | .042 | -.737 | .924 |
| Great Tit | -.919 | -.375 | -.084 | -.014 | .992 |
| Longtailed Tit | -.816 | -.049 | -.250 | .283 | .811 |
| Lesser Whitethroat | -.854 | -.039 | -.469 | .040 | .953 |
| Wren | .038 | -.902 | .370 | .115 | .966 |
| Blackbird | -.775 | -.595 | .177 | .090 | .994 |
| Song Thrush | -.599 | -.791 | .017 | .013 | .986 |
| Spotted Flycatcher | .991 | -.083 | -.015 | .048 | .992 |
| Robin | -.669 | -.713 | .128 | .096 | .982 |
| Dunnock | -.911 | -.366 | .111 | -.108 | .988 |
| Bullfinch | -.720 | .530 | -.186 | -.390 | .985 |

**Table 6.4** Component matrix of agricultural variables, Four components extracted. Percentage variation explained (%); PC1: 54.1, PC2: 14.9, PC3: 10.1, PC4: 6.3

| | Component | | | | Communalities |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | |
| Autumn Barley | -.886 | -.349 | -.086 | -.062 | .919 |
| Spring Barley | .590 | .271 | -.689 | -.183 | .930 |
| Permanent Grass | .866 | -.003 | .161 | .213 | .822 |
| New Grass | -.499 | -.561 | -.277 | -.493 | .855 |
| Linseed | -.524 | -.129 | -.384 | .622 | .825 |
| Oats | .750 | .088 | -.224 | -.312 | .717 |
| Oilseed Rape | .904 | -.284 | .056 | .115 | .914 |
| Rough Grazing | -.587 | .452 | .282 | .199 | .668 |
| Set Aside | -.510 | .776 | -.047 | -.212 | .911 |
| Potatoes | -.867 | -.095 | -.340 | .029 | .877 |
| Sugarbeet | -.964 | .031 | -.169 | -.139 | .979 |
| Total tilled land | -.377 | -.868 | -.165 | .085 | 929 |
| Wheat | -.467 | -.565 | .569 | .017 | .861 |
| Herbicide | .856 | -.245 | -.132 | -.085 | .817 |
| Fungicide | -.865 | -.205 | -.175 | .145 | .842 |
| Insecticide | .691 | -.434 | -.017 | -.235 | .722 |
| Seed-treated cereals | -.900 | .150 | -.097 | -.268 | .914 |
| Fertiliser | -.464 | -.004 | .702 | -.328 | .815 |
| Neonicotinoids | .925 | -.152 | .071 | -.018 | .884 |

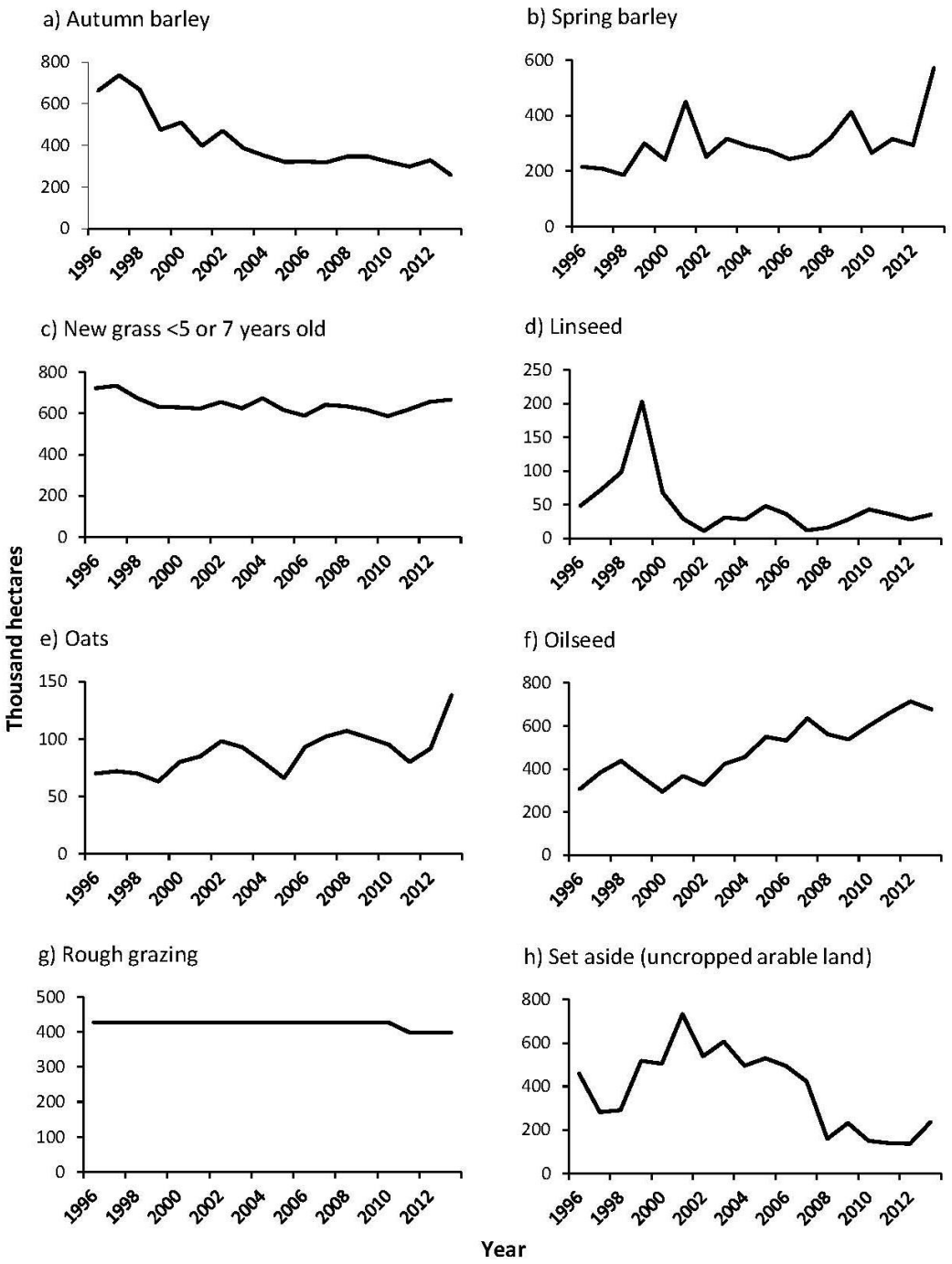
**Table S6.1** Correlation matrix of annual agricultural variables from 1996 – 2013. Pearson correlation coefficient +/- P<0.05, ++/-- P<0.01, +++/--- P<0.001.

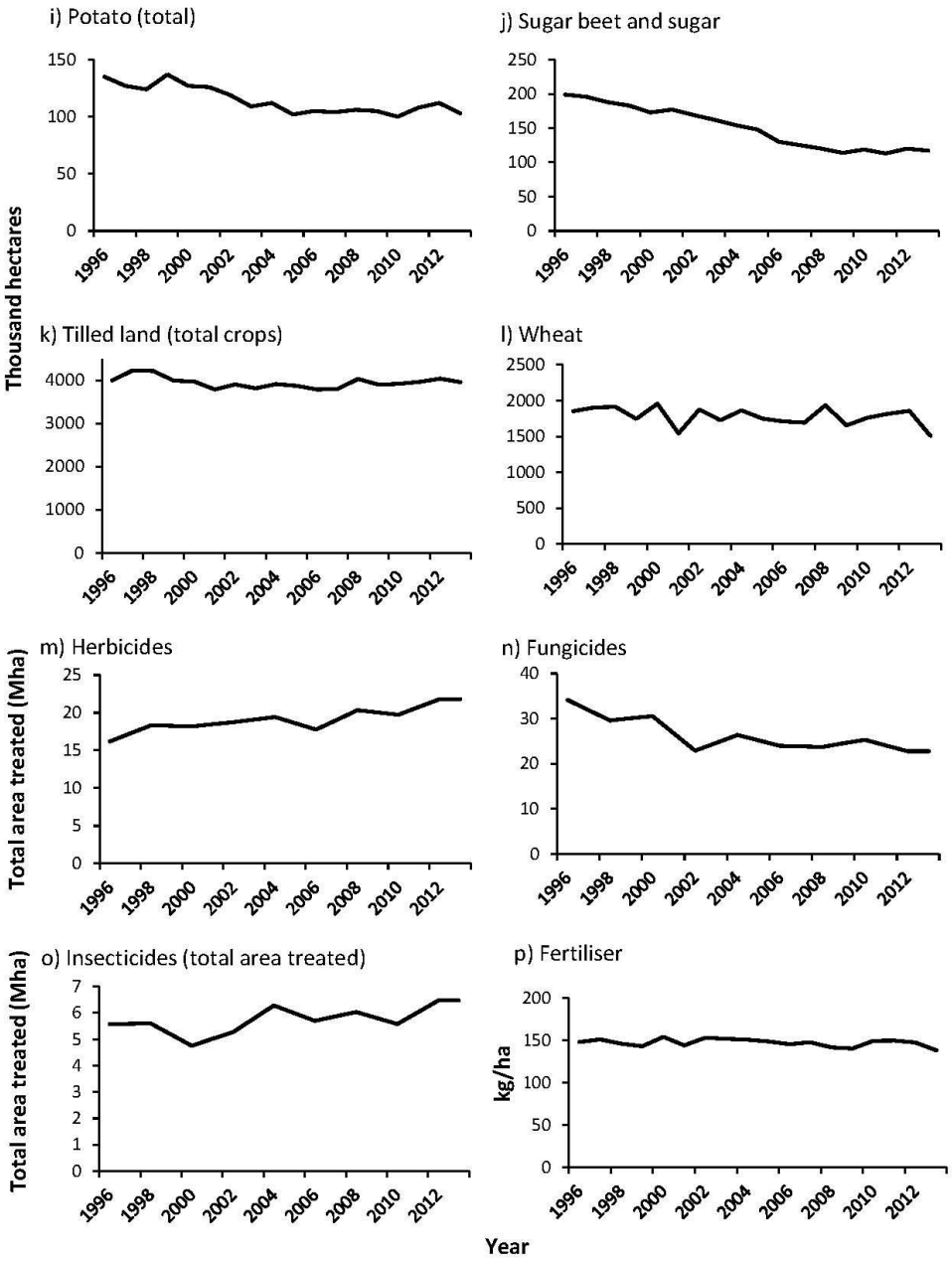
| | Autumn Barley | Spring Barley | Permanent Grass | New Grass | Linseed | Oats | Oilseed Rape | Rough Grazing | Set Aside | Potato | Sugarbeet | Tilled Land | Wheat | Herbicide | Fungicide | Insecticide | Seed Treated Cereals | Neonicotinoids | Fertiliser |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Barley** | +++ | NS | -- | ++ | NS | NS | - | NS | NS | ++ | ++ | + | NS | NS | ++ | NS | ++ | -- | NS |
| **Autumn Barley** | | - | -- | ++ | NS | - | - | NS | NS | +++ | +++ | ++ | + | -- | +++ | + | +++ | --- | NS |
| **Spring Barley** | | | NS | NS | NS | NS | ++ | NS | NS | NS | NS | NS | --- | ++ | NS | NS | NS | + | -- |
| **Permanent Grass** | | | | - | - | ++ | +++ | NS | - | --- | --- | NS | NS | + | -- | + | --- | ++ | - |
| **New Grass** | | | | | NS | NS | NS | NS | NS | + | + | ++ | NS | NS | + | NS | NS | - | NS |
| **Linseed** | | | | | | - | NS | NS | NS | ++ | + | NS | NS | NS | + | NS | NS | - | NS |
| **Oats** | | | | | | | + | NS | NS | -- | -- | NS | - | ++ | -- | NS | -- | ++ | NS |
| **Oilseed Rape** | | | | | | | | -- | -- | --- | --- | NS | NS | +++ | -- | +++ | --- | +++ | NS |
| **Rough Grazing** | | | | | | | | | + | NS | + | NS | NS | --- | NS | - | + | -- | NS |
| **Set Aside** | | | | | | | | | | NS | + | - | NS | - | NS | - | + | - | NS |
| **Potato** | | | | | | | | | | | +++ | NS | NS | -- | +++ | -- | +++ | --- | NS |
| **Sugarbeet** | | | | | | | | | | | | NS | NS | --- | +++ | -- | +++ | --- | NS |
| **Tilled Land** | | | | | | | | | | | | | ++ | NS | + | NS | NS | NS | NS |
| **Wheat** | | | | | | | | | | | | | | NS | NS | NS | NS | NS | + |
| **Herbicide** | | | | | | | | | | | | | | | --- | ++ | -- | +++ | NS |
| **Fungicide** | | | | | | | | | | | | | | | | - | ++ | --- | NS |
| **Insecticide** | | | | | | | | | | | | | | | | | -- | + | NS |
| **Seed Treated Cereals** | | | | | | | | | | | | | | | | | | --- | + |
| **Neonicotinoids** | | | | | | | | | | | | | | | | | | | NS |

**Table S6.2** Neonicotinoid exposure risk matrix. Diet information, foraging habitat (Butler *et al*. (2007) and whether the species was a specialist or generalist Robinson *et al*. (2016)) was scored and summed to produce a neonicotinoid exposure risk (NER) score. Migratory status was included to control for this in the analysis.

| | | | **Summer diet** | | | | **Winter diet** | | | | **Summer foraging habitat** | | **Winter foraging habitat** | **Farmland Specialist / Generalist** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | Family | Migrant | Below-ground inverts | Above-ground inverts | Sown crop seeds | Green crop plant material | Below-ground inverts | Above-ground inverts | Sown crop seeds | Green crop plant material | Feed in crop | Nest in crop | Feed in crop | Specialist (1) Generalist (0) | Neonic exposure risk score |
| Grey Partridge | Phasianidae | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 8 |
| Turtle Dove | Columbidae | 1 | 0 | 0 | 1 | 1 | | | | | 1 | 0 | | 1 | 4 |
| Rook | Corvidae | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 10 |
| Skylark | Alaudidae | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 9 |
| Starling | Sturnidae | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 8 |
| Yellow Wagtail | Motacillidae | 1 | 0 | 1 | 0 | 0 | | | | | 1 | 1 | | 0 | 3 |
| Linnet | Fringillidae | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 5 |
| Yellowhammer | Emberizidae | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 6 |
| Corn Bunting | Emberizidae | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 7 |
| Spotted Flycatcher | Muscicapidae | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 1 |
| Lapwing | Charadriidae | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 7 |
| Stock Dove | Columbidae | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 6 |
| Greenfinch | Fringillidae | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 5 |
| Chaffinch | Fringillidae | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 4 |
| Blue Tit | Paridae | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wren | Troglodytidae | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Song Thrush | Turdidae | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 5 |
| Robin | Muscicapidae | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Woodpigeon | Columbidae | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 6 |
| Jackdaw | Corvidae | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 10 |
| Whitethroat | Sylviidae | 1 | 0 | 1 | 0 | 0 | | | | | 1 | 0 | | 0 | 2 |
| Tree Sparrow | Passeridae | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 6 |
| Goldfinch | Fringillidae | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 5 |
| Reed Bunting | Emberizidae | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 5 |
| Great Tit | Paridae | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Long-tailed Tit | Aegithalidae | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Lesser Whitethroat | Sylviidae | 1 | 0 | 1 | 0 | 0 | | | | | 0 | 0 | | 0 | 1 |
| Blackbird | Turdidae | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 5 |
| Dunnock | Prunellidae | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Bullfinch | Fringillidae | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |


a) Autumn barley
b) Spring barley
c) New grass <5 or 7 years old
d) Linseed
e) Oats
f) Oilseed
g) Rough grazing
h) Set aside (uncropped arable land)
Thousand hectares
Year


i) Potato (total)
j) Sugar beet and sugar
k) Tilled land (total crops)
l) Wheat
m) Herbicides
n) Fungicides
o) Insecticides (total area treated)
p) Fertiliser
Thousand hectares
Total area treated (Mha)
Total area treated (Mha)
kg/ha
Year

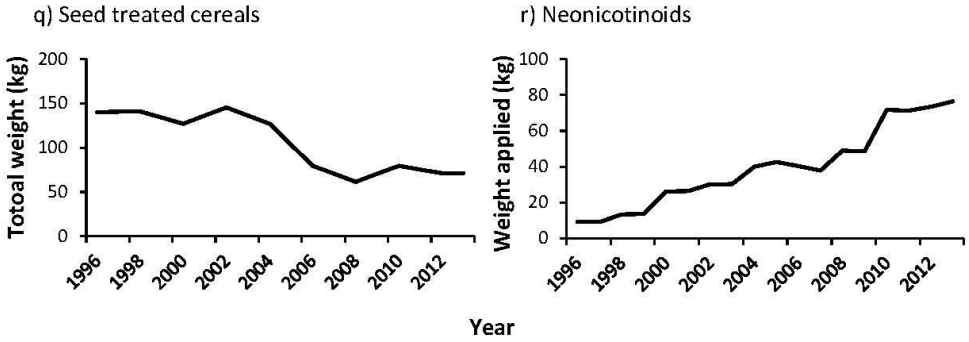



**Figure S6.1** Smoothed trends in agricultural variables in England. 4235-H smoothing has been applied to the data in order to view underlying trends.

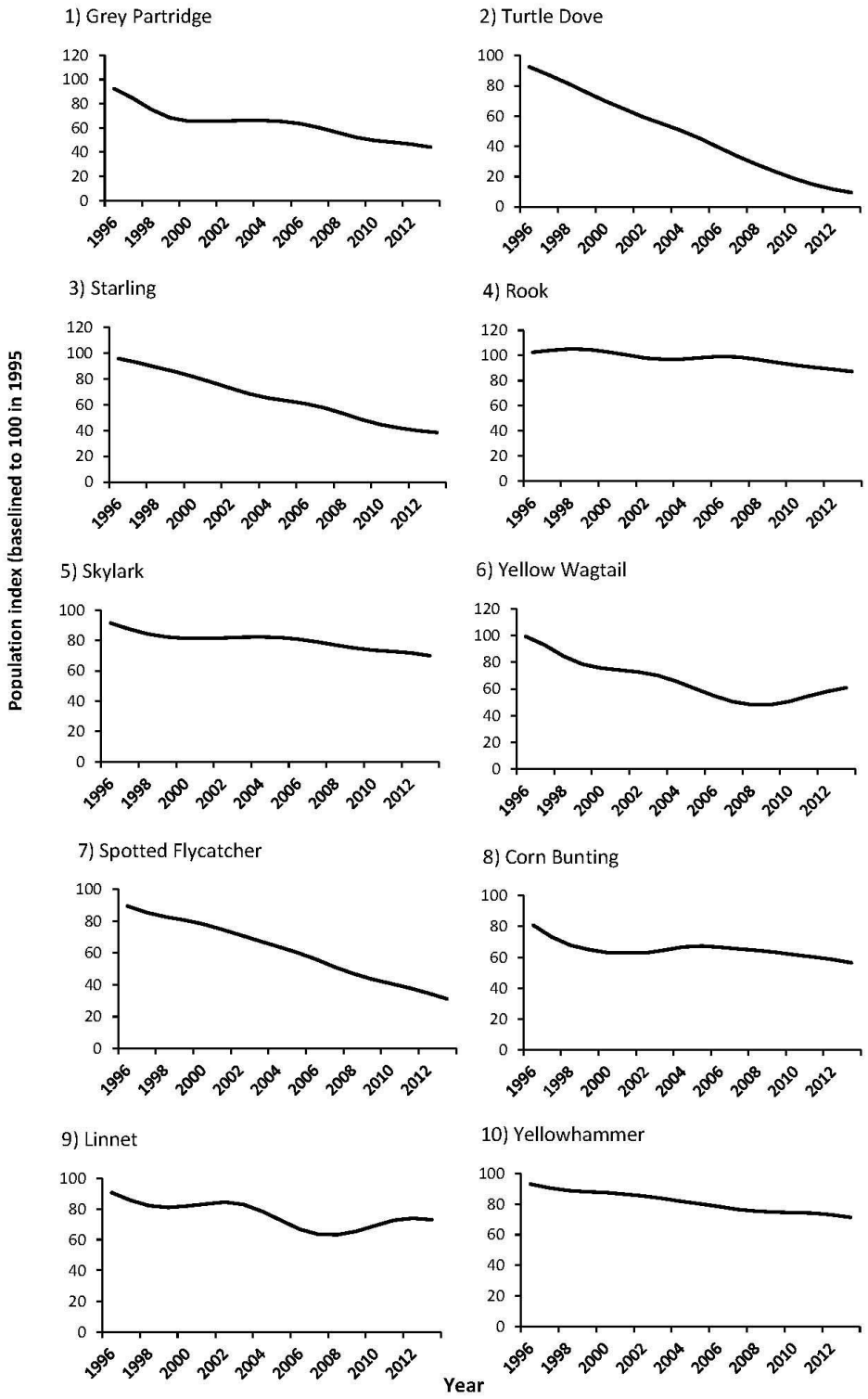
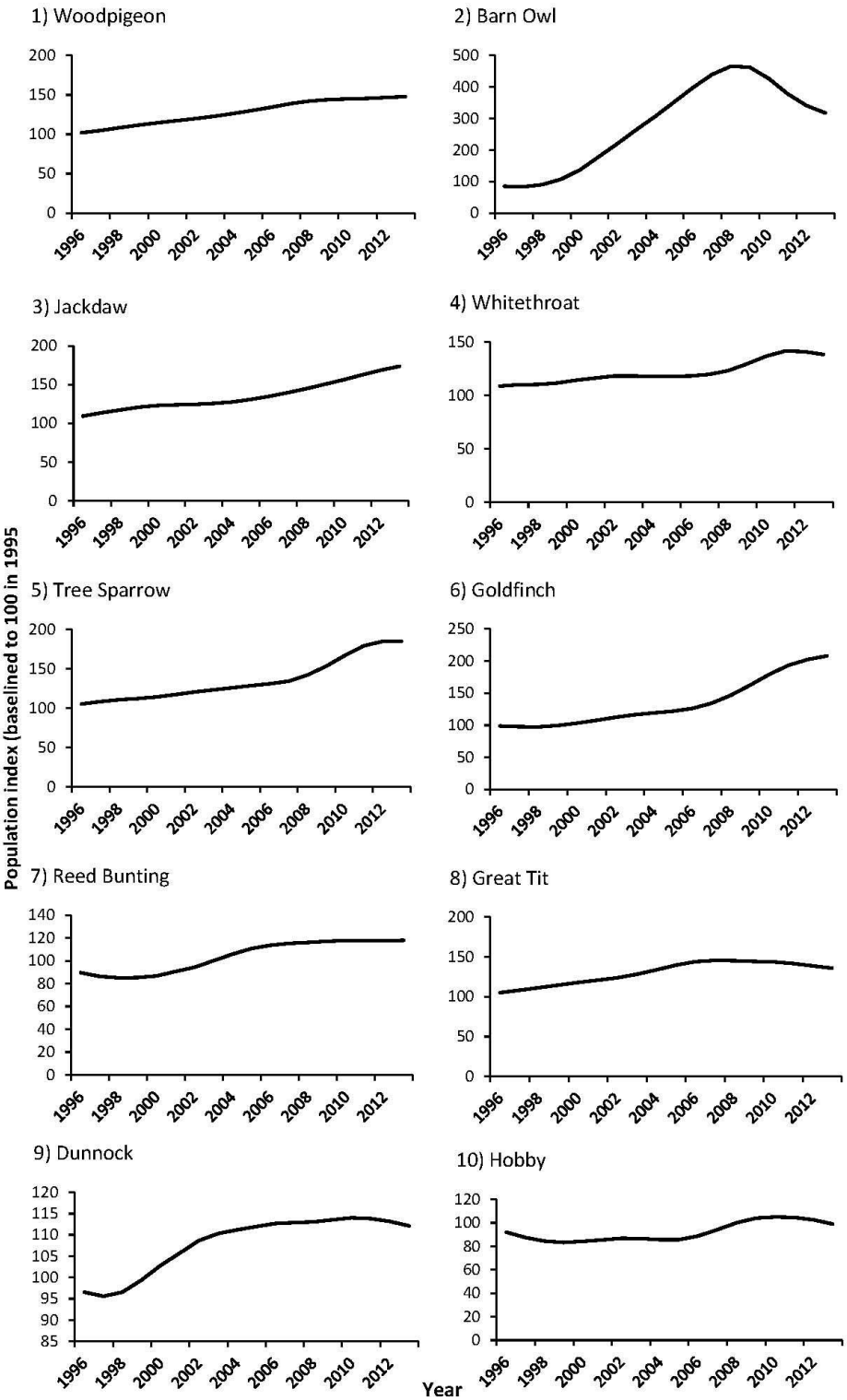
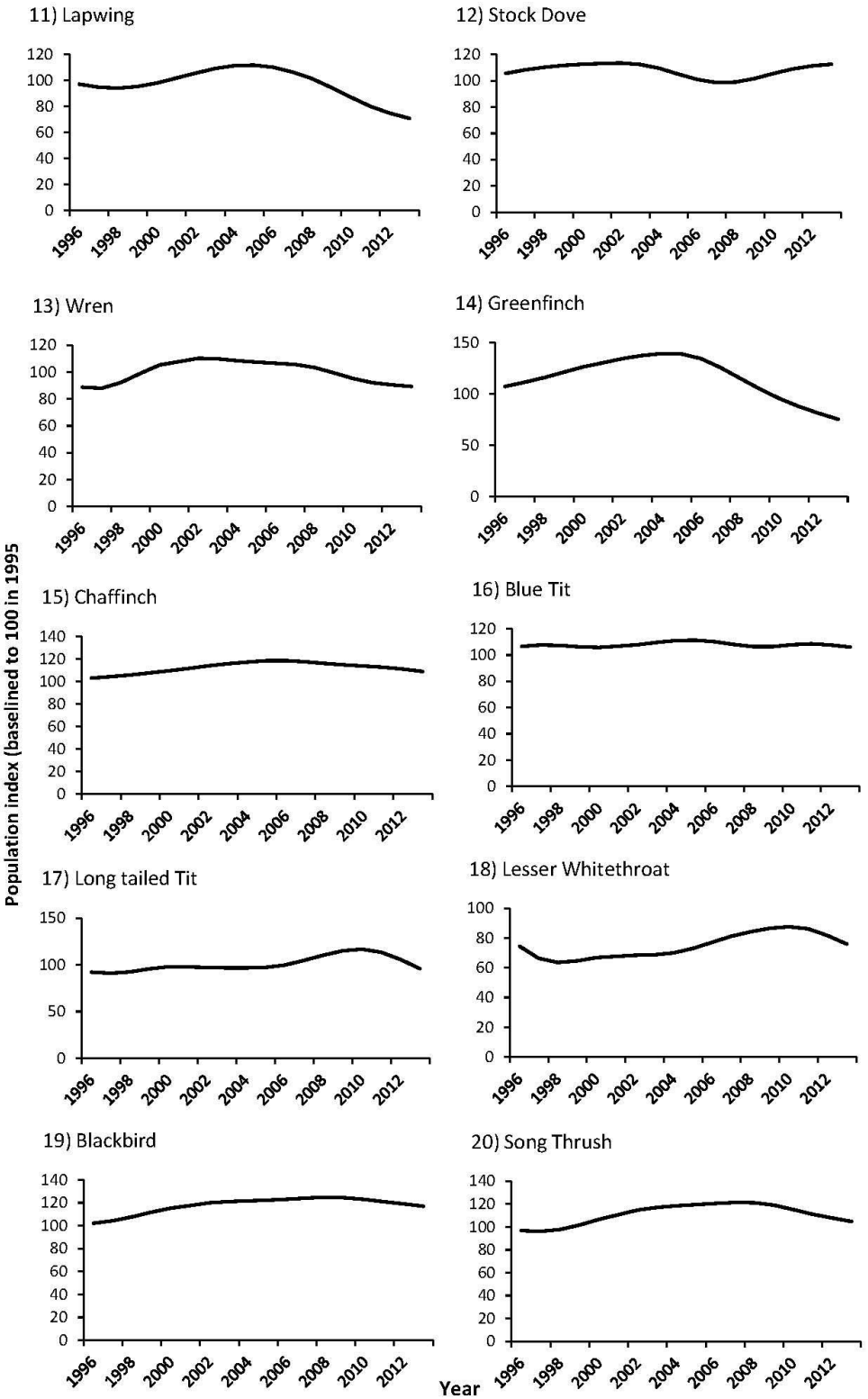



**Figure S6.2a** Smoothed population trends of steadily decreasing species used in analysis. 1) Grey Partridge 2) Turtle Dove 3) Starling 4) Rook 5) Skylark 6) Yellow Wagtail 7) Spotted Flycatcher 8) Corn Bunting 9) Linnet 10) Yellowhammer


1) Woodpigeon
2) Barn Owl
3) Jackdaw
4) Whitethroat
5) Tree Sparrow
6) Goldfinch
7) Reed Bunting
8) Great Tit
9) Dunnock
10) Hobby
Population index (baselined to 100 in 1995
Year


11) Lapwing
12) Stock Dove
13) Wren
14) Greenfinch
15) Chaffinch
16) Blue Tit
17) Long tailed Tit
18) Lesser Whitethroat
19) Blackbird
20) Song Thrush
Population index (baselined to 100 in 1995
Year

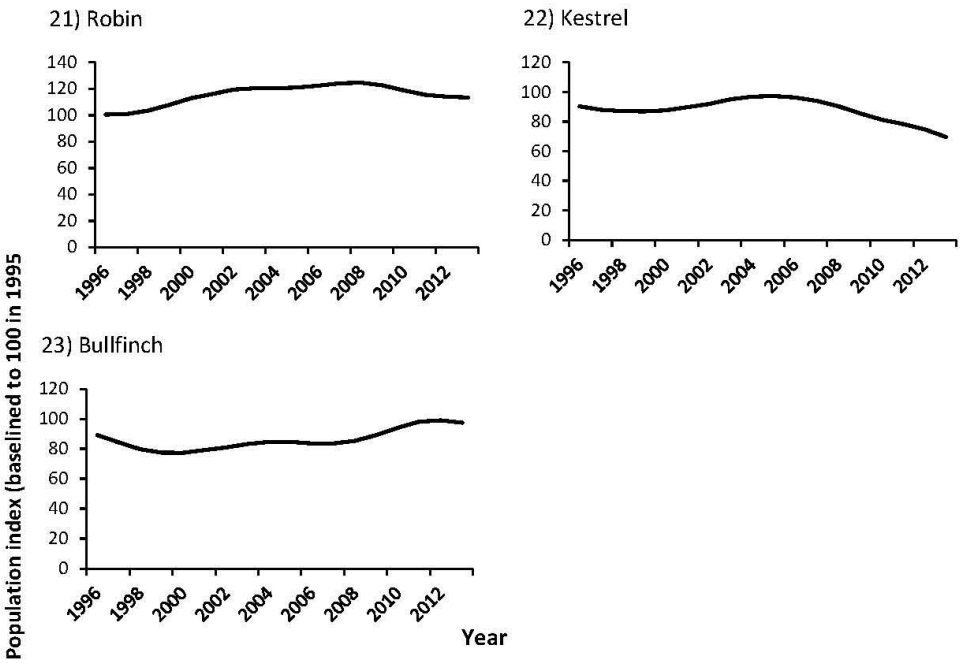



**Figure S6.2b** Smoothed population trends of increasing or stable species used in analysis. 1) Woodpigeon 2) Barn Owl 3) Jackdaw 4) Whitethroat 5) Tree Sparrow 6) Goldfinch 7) Reed bunting 8) Great tit 9) Dunnock 10) Hobby 11) Lapwing 12) Stock Dove 13) Jackdaw 14) Greenfinch 15) Chaffinch 16) Blue Tit 17) Long-tailed Tit 18) Lesser Whitethroat 19) Blackbird 20) Song Thrush 21) Robin 22) Kestrel 23) Bullfinch

# **Chapter 7** – General discussion

## 7.1 Summary of studies and outcomes

A considerable body of recent research has demonstrated that neonicotinoid pesticides have a range of detrimental effects on bees (e.g. Gill *et al*., 2012; Whitehorn *et al*., 2012; Di Prisco *et al*., 2013; Gill & Raine, 2014, Rundlof *et al.* 2015, Woodcock *et al.* 2017). However, their impact on other non-model organisms is less well understood, despite these species fulfilling equally vital ecosystem functions. The projects described throughout this thesis thus focused on determining the impact of chronic exposure to field relevant levels of the neonicotinoids thiamethoxam and clothianidin on ecologically important non-model organisms (organisms that have not been selected for extensive research); specifically, those species typically found in agroecosystems, and therefore those most likely to come into contact with neonicotinoids. Three approaches were taken to address this gap in knowledge:

i) Laboratory-based experiments, to establish novel model systems for three different organisms across a diverse series of taxa: the hoverfly *Eristalis tenax*, the butterfly *Polyommatus icarus*, and the earth worm *Lumbricus terrestris* (Chapters 2, 3, & 5 respectively).

ii) A semi-field experiment, to investigate the colonisation of contaminated microcosms by aquatic invertebrates (Chapter 4).

iii) Analysis of time-series data from 1996 – 2013, to assess the relationship between shifts in agricultural practices, including the degree of neonicotinoid usage, and changes in the population of multiple farmland bird species.

My major original contributions to current understanding of the impact of neonicotinoids on non-target organisms are as follows:

a) The larval stage of the hoverfly *Eristalis tenax* was unaffected by field realistic doses of thiamethoxam. There were no observed effects on survival, development, nor any latent effects on adult activity budgets resulting from exposure to lower concentrations (up to 100 ppb). Increased mortality in larvae was observed at 500 ppb, however this level is unlikely to be encountered by hoverflies in the field (*Chapter 2*).

b) Clothianidin negatively affected the survival of *Lumbricus terrestris*, when exposed via treated soil only. There was no clear effect when clothianidin was introduced via soil and food, or food only. Field-realistic exposure had a significant, but temporary effect on food consumption; there was an anti-feedant effect for 2 months which subsequently disappeared (*Chapter 3*).

c) Both clothianidin and thiamethoxam had significant negative effects on Chironomids (Diptera) and Ostracoda in aquatic microcosms, and there were clear differences between the effects of each chemical. Field realistic concentrations of neonicotinoids are likely to reduce populations of insects found in ephemeral ponds, which may affect trophic interactions (*Chapter 4*).

d) Larvae of *Polyommatus icarus* exhibited varying responses to clothianidin exposure, including both lethal and deleterious sub-lethal impacts. Exposure to 15 ppb or above reduced larval growth for the first 9 days of the experiment. Although there was evidence of clothianidin inducing mortality in larvae, with highest survival in control groups, the dose−response relationship was unclear. Non-target herbivorous organisms living in arable field margins are also likely to be chronically exposed to neonicotinoids due to the contamination of foliage from contaminated soil. (*Chapter 5)*

e) A strong correlation was found between the principal components of agricultural change and changes in populations of farmland birds. This is consistent with the hypothesis that agricultural change continues to affect farmland bird populations. Neonicotinoid usage was also included as a variable of agricultural change, and a moderately significant relationship was identified specifically between bird population change and neonicotinoid exposure risk score ('NER' – calculated by combining direct adverse effects, due to consumption of contaminated seeds and leaves and indirect effects via depletion of insect prey) (*Chapter 6*).

The question of how neonicotinoids impact on farmland wildlife continues to be an active area of research with high policy relevance (Godfray *et al*., 2015, EFSA 2018). The above studies are complementary with previous research on non-target organisms and neonicotinoids, demonstrating that neonicotinoid exposure at field-relevant levels can have negative effects on mortality, food consumption and growth across a range of organisms – although not all organisms are affected equally. It further highlights the problems and solutions encountered when trying to provide field-realistic data for use in assessing risks posed by use of neonicotinoids, via laboratory experiments.

## 7.2 Environmental relevance of research

There is a growing body of evidence suggesting that neonicotinoids produce population-level impacts at field-observed levels (Van Der Sluijs *et al*., 2015). Extensive use of neonicotinoids over recent years (DEFRA, 2017b), combined with their high water solubility and environmental persistence has led to widespread contamination of both terrestrial and aquatic natural resources (particularly soils, waterways and non-treated vegetation) at levels that are known to cause lethal and sub-lethal effects in a wide range of organisms (Van Der Sluijs *et al*., 2015). This contamination is likely to affect the ecological functions and resilience of these environments, and impact the ecosystem services provided to humans and the biosphere; including soil and freshwater functions, fisheries, biological pest control, and pollination services (Van Der Sluijs *et al*., 2015). The studies included in this thesis further demonstrate that neonicotinoids have an impact on biodiversity in both soil and water.

### *7.2.1 Soil contamination*

Soil decomposer food webs play an essential role in key ecosystem processes: soil formation, nutrient cycling and soil carbon storage (EASAC, 2015). Yet, temporal trends (though limited) show soil biodiversity levels to be potentially under high pressure in ~23%, and very high pressure in 8%, of the surface area of the European Union (excluding Finland and Sweden) as a result of current levels of land use change, habitat disruption, impacts of invasive species, soil compaction, erosion and pollution (EASAC, 2015). The biodiversity of soil food webs was examined across agricultural regions in Europe and it was found that intensive agriculture made soil food webs less diverse and composed of smaller bodied organisms (Tsiafouli *et al*. 2015).

Neonicotinoids can persist in soil for long periods (months to years), so have a great potential for adverse effects on soil ecosystems (Chagnon *et al*., 2015). Furthermore, it is thought that invertebrate-mediated soil processes are at greater risk of negative effects from neonicotinoid residues than microbial-mediated processes (Chagnon *et al*., 2015). Despite this recognised risk of harm, few previous studies have looked at the effects on the ecologically relevant test species that contribute to soil ecosystem functions, such as earthworms. Due to their unique and major role in the contribution to soil health, earthworms occupy a very important niche (Tomlin, 1992). Their wide and deep-penetrating burrows open up the structure of compacted and clay soils by enabling water infiltration (Nuutinen, Butt & Jauhiainen, 2011), and soil fertility is enhanced by the breakdown of plant litter and the mixing of this litter with the soil (Pisa *et al*., 2015).

The effects of chronic exposure to clothianidin on the common earthworm *L. terrestris* were investigated in Chapter 3. Although no effects on body mass or mortality were found following clothianidin exposure, a significant negative effect was seen on the consumption or collection of contaminated food during the first two months of the study. This corroborates previous findings that field-rate applications of clothianidin can retard grass clipping decomposition in the long-term (Larson *et al.*, 2012). Interestingly, there was a recovery in the rate of food consumption towards the end of the experiment, but it is unclear if this was due to desensitisation to the chemical, or feeding inhibition being overridden by hunger. If this recovery also occurs in the field, it is possible that clothianidin exposure may not have long-lasting adverse effects on the role earthworms play in the soil ecosystem.

My data provide evidence that neonicotinoids can impair some soil ecosystem services in the short term. However, since neonicotinoids have been found to bioaccumulate in the earthworm *Eisenia andrei* (Chevillot *et al*., 2017), and little is known about the trophic effects of consuming contaminated prey on organisms – such as birds – which are higher up the food chain, longer-term multiple-chemical exposure studies are needed to determine the lasting effects of exposure on ecosystem functioning.

### *7.2.2 Contamination of vegetation*

Alongside direct exposure, the persistence of neonicotinoids in the environment poses an additional risk to non-target organisms via their exposure to non-crop contaminated vegetation – such as those wildflowers found in field margins and planted flower strips.

Wild and planted non-crop flowers are a vital component of the agricultural landscape because they can enhance crop yield, support wildlife biodiversity, and provide habitat for natural predators of pests; they are therefore often the target of agri-environment schemes designed to maintain these benefits (Botías *et al*., 2016). Research into the efficacy of such schemes is ongoing – such as the UK study by the Centre for Ecology and Hydrology, investigating wildflower strips planted throughout the crop instead of only around the field's perimeter (CEH, 2018). As well as monitoring the effects on crop yields, a substantial area of research is focused on the ability of wildflower planting schemes to draw beneficial predatory and pollinating insects out into the field, closer to the crops.

However, field margins are easily contaminated with pesticides that are used on the fields themselves. Chapter 5 details an experiment where the levels of clothianidin in non-target field margin vegetation was tested under two different treatment scenarios. The results

suggest that the contamination of vegetation by clothianidin may be more likely when wildflowers are planted directly into soil that has previously supported a crop grown from neonicotinoid-treated seed, compared to contamination levels of a flower margin grown alongside a treated crop. These preliminary results are comparable to work by Botías *et al.* (2016) who found levels of neonicotinoids in foliage from non-target plants growing in the crop field margins to be in the range of ≤0.02–106 ng/g; furthermore, the maximum contamination levels in the field margin vegetation commonly exceeded the $LC_{50}$ values reported in the literature for a set of non-target, important insects (Botías *et al*., 2016). Together, these results indicate that the contamination of those areas through unintended movement of neonicotinoids throughout the environment is greater than previously thought, with potentially large knock-on effects on wildlife. If farmers are to adopt the use of wildflowers in this manner more in the future, precautionary measures should be put in place to ensure that these flower-strips (if grown in neonicotinoid-contaminated soil) do not lead to the exposure of beneficial insects to harmful levels via wildflower pollen, nectar, and foliage. This may include regularly turning the soil to expose the full profile to UV light, which is known to degrade neonicotinoids (Goulson, 2013), combined with regular testing to monitor the levels of neonicotinoids before planting.

### *7.2.3. Effects on the butterfly Polyommatus icarus of exposure to contaminated vegetation*

A recent correlational study modelled the population indices of 17 widespread butterfly species that commonly occurred at farmland sites, against the number of hectares of farmland where neonicotinoid pesticides are used (Gilburn *et al*. 2015). Fifteen of the seventeen species studied showed negative correlations with neonicotinoid usage; the butterfly examined in Chapter 5 of this thesis (*Polyommatus icarus)* underwent a 30% decline from 2000 to 2009, showing a weak negative relationship with hectares of neonicotinoid usage.

In my study, while larval exposure to a field realistic clothianidin dose (5 ng/g to 50 ng/g used in the experiment, based on field measures of 48ppb clothianidin) had no impact on mortality of *P. icarus*, the contamination of food with clothianidin had a detrimental effect on early stage development, with larval size being reduced by the presence of field realistic levels. These data add to growing evidence that the pollution of non-target vegetation may be detrimental to early herbivorous larval stages. Clothianidin had no effect on the time to pupation, due to apparent compensatory growth in the later stages; combined with Gilburn *et al.*'s findings, this suggests that *P. icarus* may be overall less sensitive to clothianidin exposure

than other butterflies, but further experimental studies to determine the toxicity and effect of other neonicotinoids on *P. icarus* adults are required.

Chapter 5 also details the novel methods developed and used in my *P. icarus* studies. Previous works utilised a leaf-dip assay or an agarose gel containing the contaminant (Brunner *et al*., 2005; Pecenka & Lundgren, 2015), but due to the nature of the exposure route, larvae can be exposed to the contaminant by contact residues as well as orally. To ensure larvae were only exposed via the consumption of the foodstuff, a novel contamination method was designed, allowing for reporting of only the effects of oral toxicity; plants were placed in trays containing the solution, through a cardboard spacer to avoid UV degradation of the clothianidin, and plants took up the solution via their roots only. This method also mimics natural exposure methods much closer than a leaf bioassay or artificial lepidopteran food. The contamination technique resulted in vegetation that was very closely matched in neonicotinoid concentration to that of the watering solution.

In addition, much of the research on the impact of neonicotinoids on herbivorous larvae has used short exposure durations, with the maximum being 7 days, and the longest specific clothianidin exposure in the available literature at 36hrs (Pecenka & Lundgren, 2015). It has been argued that studies with short exposure durations may miss the critical period where a time-cumulative effect of the toxin is detected (Rondeau *et al*., 2014). My study followed the larvae from eggs through to metamorphosis and emergence, with chronic exposure occurring from when larvae were one week old. Due to the larvae's small size it is unlikely that they will disperse from the field margin (Weiss & Murphy, 1988), and therefore a chronic oral exposure would be expected, so this technique was developed to better simulate that route.

### *7.2.4 Water contamination*

Any reduction in the growth, survival, abundance or reproduction of aquatic insects and crustaceans can alter ecosystem functions related to decomposition and nutrient transfer from primary producers to consumers (Chagnon *et al*., 2015), effectively damaging the underlying insect communities that provide a rich food source not only to fish, but also birds and other insectivorous vertebrates (Pisa *et al*., 2017). Water on farmland, including surface waters, puddled water, ditches, irrigation channels and streams have been found to be contaminated by neonicotinoids (Van Dijk, *et al*., 2013; Main *et al*., 2014; Samson-Robert *et al*., 2014; Morrissey *et al*., 2015; Schaafsma *et al*., 2015). A recent report by Buglife (a UK invertebrate conservation charity) using results from the EU Water Framework Directive, found that half of

the rivers monitored in England exceeded chronic pollution limits for neonicotinoids and two rivers were acutely polluted (Shardlow, 2017).

Further, the chronic exposure to low levels of neonicotinoid residues in water is regarded as causing a long-term lethality in most aquatic species, which eliminates entire populations from the affected areas (Pisa *et al*., 2017). Work by Hallmann *et al*. (2014) have already demonstrated that depletion of insect food resources caused by pollution of aquatic habitats had a negative impact on insectivorous passerine bird species in the Netherlands. The risks associated with the knock-on and potential cascading effects of a neonicotinoid presence in freshwater are present across the globe wherever neonicotinoids are being used, and indicates a global failure of pesticide regulation. The current assumption underlying pesticide regulation—that chemicals that pass a battery of tests in the laboratory or in field trials are environmentally benign when they are used at industrial scales – is false (Milner & Boyd, 2017).

The study in Chapter 4 considered the effects of neonicotinoid contamination on the colonisation of water bodies by invertebrate populations. Significant negative effects were shown resulting from exposure to the neonicotinoids thiamethoxam and clothianidin. Although both showed negative effects, there were also clear differences between the two chemicals, with thiamethoxam generally producing stronger negative effects than clothianidin. Populations of Chironomids (Diptera) and Ostracoda were negatively affected by both chemicals, while Culicidae appeared to be unaffected by clothianidin at the doses used. These data corroborate existing research on other neonicotinoids; for example, imidacloprid has had demonstrable negative effects on aquatic invertebrate life (Van Dijk *et al*., 2013).

The effects of aquatic pollution by thiamethoxam was also examined in Chapter 2 for impacts on the larvae of the hoverfly *Eristalis tenax*. Survival rates were significantly decreased by exposure to 500 ppb thiamethoxam, but this concentration exceeds that likely to be found in the field. There was no observed effect on *E. tenax* survival, development, or any latent effect on adult activity budgets resulting from exposure to lower concentrations (up to 100 ppb). This is an encouraging discovery, considering that many studies show significant harm to other pollinators from neonicotinoids (Rundlöf *et al*., 2015; Tsvetkov *et al*., 2017; Woodcock *et al*., 2017), and highlights the importance of testing a wide-range of study organisms (particularly biologically-relevant species that may be exposed via contaminated habitats). However, we have little idea as to the mechanisms explaining why toxicity should vary so greatly between species. Species' life-history traits can influence their vulnerability to pesticides, and different

endpoints can substantially differ in sensitivity even within species (Arena & Sgolastra, 2014). Further investigation into different endpoints for *E. tenax* is required to determine possible adverse effects on adults from exposure to contaminated food.

### 7.3 The impact of changing neonicotinoid use and other farming practices on farmland birds and declining insect biomass

Concerns have been raised about the negative impacts of neonicotinoids on trophic interactions and trophic structure (Chagnon *et al*., 2015, Hallmann *et al*., 2014), especially on the impact on farmland birds either via direct toxicity or reduction of food resources (Gibbons *et al*., 2015). Undertaking ecotoxicological laboratory work on multiple bird species is neither practical, nor deemed ethical. However, analysing patterns and interactions in time-series data is a useful tool.

Generally, correlative studies using time series data cannot prove causative relationships of the factors driving population change, but can be used to identify possible drivers of change, and support evidence from experimental studies. The study outlined in chapter 6 updated an analysis by Chamberlain *et al*. (2000) looking into the potential impacts of neonicotinoid usage (and other farming changes) on the populations of farmland birds, by testing the relationship between multivariate changes in agricultural practise and multi-species trends in bird populations on farmland in England for the period 1996-2013. As neonicotinoid pesticides are a rapidly-increasing component of arable agricultural practice in England (since the mid-1990s), the statistical testing focused on whether there was any association between variation in bird species population trends and variation in likely exposure of different species to any direct or indirect impacts of neonicotinoid use, based on species' ecological traits, used to calculate their neonicotinoid exposure risk score (NER).

Neonicotinoid usage emerged as the most persistent and rapidly changing agricultural practice over the period 1996 – 2013. The declining bird species tended to be those with higher NER scores, which suggests that more detailed studies of the potential direct, toxic impacts of neonicotinoids on granivorous species, and the potential indirect effects via reduction in invertebrate food supply on insectivorous species, are worthy of more detailed study. This recommendation is further strengthened by comparison with the findings of Hallmann *et al*. (2014) who found significant negative relationships between surface-water imidacloprid concentrations and population trends for eight of fifteen bird species studied in the Netherlands (after correcting for spatial differences in land-use changes known to affect farmland bird populations).

Most avian studies have focused on the direct acute toxic impacts of neonicotinoid consumption via treated seed (Hallmann *et al*., 2014; Pisa *et al*., 2015, 2017). Evidence of indirect, food chain effects of neonicotinoids remains rare (Pisa *et al*., 2017), but more studies are being published detailing the devastating decline of insects, which form the basis of hundreds of food chains. A recent study by Hallmann *et al*. (2017), based on data collected from 63 protected sites in Germany, found that the number of flying insects has plummeted by more than 75 percent in the last 25 years: a truly alarming and dramatic decline. This study found that the decline was independent of habitat type, changes in weather or land use, and changing habitat characteristics alone could not explain the overall decline. Agricultural intensification could not be incorporated into the analysis, but is suggested as being an aggravating factor in the reduction of insect biomass (Hallmann *et al*., 2017). A loss of insect diversity and abundance is expected to cause cascading effects on food-webs and ecosystem services (Hallmann *et al*., 2017, Ewald *et al*., 2015, Fox 2013).

Studies like Hallmann *et al*. (2017) can only happen because of access to the data collected by widespread, standardised sampling of invertebrates, often performed by dedicated amateur entomologists. Unfortunately, long-term records are often infrequent and ad hoc, with no standardised sampling programs in place for the large majority of insect taxa. Even for taxa with a recognised economic contribution such as pollinators (except for butterflies, due to the UK Butterfly Monitoring Scheme and European schemes like the eBMS) available data is fragmentary, due to a lack of coordinated monitoring programmes (Potts *et al*., 2010). To address the imbalance between the available biodiversity data and the increasing need for such records, long-term monitoring schemes for many more species groups are required. In the current economic climate funding is likely to be restricted. There is potential in the rising area of formalised 'Citizen Science' methods, which can be cost effective but would need substantial co-operation between governmental organisations, farming organisations, academia, charities and trusts to adopt a unified approach.

### 7.4. Challenges of conducting effective field-realistic experiments

There are typically many caveats given about the relevance of environmental research, and many of these stem from limitations generated by replicating field realistic scenarios in the laboratory. The advantages of laboratory experiments are clear: they provide a controlled environment, with accurate measurement of concentrations and effects (EASAC, 2015). However, they are also often criticised for being unrealistic, often due to the conditions of the experiment being unreflective of conditions in the wild (Walters, 2013). Further, much of the

available literature about neonicotinoids impacts is based on short term, acute exposure scenarios which do not reflect the typical exposure window in the field, which is why it was important to undertake chronic exposure studies.

The life stage at which the exposure began may also affect the response of the animal to the contaminant. The different experiments commenced with different ages of animal: the *E. tenax* experiment commenced with five day old larvae, the *P. icarus* experiment with seven day old larvae, and the *L. terrestris* with mature worms with clittelum. This was essential to allow handling of the animal. Earlier life stages (larvae specific) have been consistently shown to be more sensitive to contaminants due to differences in biomass and bioaccumulation after exposure to a contaminant (Heinis *et al*. 1990). It is possible that if these experiments had commenced with eggs being laid directly either in contaminated water and soil, or directly onto contaminated foliage, hatching or commencement of growth could be more adversely affected.

It was also important to use relatively-common organisms that would be found in areas likely to become contaminated by neonicotinoids, and that would be relatively easy to maintain under laboratory conditions. The studies undertaken for this thesis involved setting up experimental systems for non-model organisms, and the application of chemicals in isolation. Though focusing on single chemicals allowed for stronger conclusions to be drawn under novel rearing conditions of non-model organisms, exposure to multiple chemicals at once can be argued to be more realistic and hold more environmental relevance, but are extremely challenging to undertake. However, since these experiments were intended to reflect possible effects of neonicotinoids in the field, the challenges of making and maintaining appropriate conditions, as well as experimental limitations, should be acknowledged.

The effect produced by combinations of chemicals applied in 'real' field situations is always a concern. Agrochemicals are often sold and applied in combination, even if the formulations are separate, so we would expect bees and other non-target organisms to be simultaneously exposed to a complex mixture of agrochemicals (Botias *et al*., 2015; Hladik *et al.*, 2018). In general, research and risk assessments typically only evaluate the risk of harm from single active substances, and considers mixtures only when the compounds are part of the same formulation (Botías *et al*., 2017). However, numerous formulations are typically applied during the same cropping season (Garthwaite *et al*. 2013; Botias *et al*. 2015), including fungicides – which have recently and unexpectedly been found to be the strongest predictor of range contractions in declining bumblebee species (McArt *et al*., 2017). Multiple compounds can act

additively, as synergists, or as antagonists and can co-occur with other contaminants such as heavy metals and fertilisers (Hladik *et al.*, 2018). Multiple compounds are detectable in pollen, nectar and bee tissue (Giorio *et al*. 2017), offering robust evidence of their pervasiveness in the environment. Research into combined effects is a very important ongoing area, beyond the scope of this investigation.

Also important to consider is the method of exposure of study organisms to the focal contaminant, and whether the doses are relevant to the field (Godfray *et al*., 2015). The dose insects receive in real-world situations is hard to predict; for example, the insect could be exposed to the chemical but also forage elsewhere on an uncontaminated resource; or may avoid contaminated food or surroundings when given other options. This is particularly true of adults, which tend to be far more mobile than immature stages. Conducting full-field experiments with highly mobile organisms such as flying insects is particularly challenging and would require complex protocols and collaboration with agricultural centres and farms who had the space to accommodate such investigations. Since my studies focused on the effect of the chemicals, the animals were not given a choice in diet or situation, to ensure that defined environmental conditions were maintained. It would be possible to extend the laboratory and semi-field studies into full field-realistic trials, if the results of these constrained investigations suggested significant enough effects to warrant expansion of the protocol, but considering the high resource requirements needed to do so, the importance of conducting initial investigations even in 'unrealistic' laboratory settings should not be downplayed.

Even though we had assurances from ground staff that neonicotinoids were not used on university grounds, it is still possible, though very unlikely, that the substrates provided to the hoverfly larvae (Chapter 2) and the soil for the aquatic microcosms (Chapter 4) could have been contaminated with neonicotinoids. We did not have the funds or capacity to run these samples through GCMS to be absolutely certain that they were neonicotinoid free, but we considered the site records to provide sufficient confidence to proceed with experiments. Similarly, while pollen and loam purchased from outside suppliers was sold as free of pesticides, funding constraints meant that we were not able to double check this.

### 7.5 Alternatives to neonicotinoids

Some studies suggest that use of chemical pesticides are essential for the control of pests, and that a reduction in their use may cause a drastic loss of farm profits and crop yields (Cooper & Dobson, 2007; Jess *et al*., 2014). Others claim a reduction in use could result in increased crop productivity and profit, such as the 2017 study by Lechenet *et al*., which demonstrated that

low pesticide input rarely resulted in a decrease in productivity and profitability on arable farms in France; 77% of the farms were found to be more profitable overall with an average reduction of 37%, 47% and 60% of herbicide, fungicide and insecticide use, respectively. Similarly, the Environmental Protection Agency in the United States concluded that the use of neonicotinoids seed treatments for insect control in soybean production provided little or no overall benefits in most situations. Published data indicated that in most cases there was no difference in soybean yield when soybean seed was treated with neonicotinoids versus not receiving any insect control treatment (EPA 2014). Further, the intensive use of insecticides over many years leads to pest populations that are resistant to several classes of this group of chemicals. Bass *et al.* (2014) described a case study focusing on *Myzus persicae* and its remarkable ability to overcome toxic effects of insecticides. The work on *M. persicae* also reinforces the dangers of widespread and long-term reliance on a select few insecticide classes, including neonicotinoids, to which they are also resistant. Future pest-control strategies need to use insecticides in carefully tailored, local, management plans in order to control resistance development in highly adaptable pest species (Bass *et al*., 2014).

Integrated pest management (IPM) programs are designed to optimise efficacy of biocontrol agents whilst simultaneously minimising insecticide effects on biological control (Casida, 2012). One of the most important pieces in the IPM framework is the process of scouting for disease and pesticides and reacting accordingly. As most seeds are prophylactically treated with pesticides, including neonicotinoids, the subsequent application of treatments only in response to pests is currently impossible (Tooker *et al.,* 2017; Goulson, 2013). IPM practices are only compatible with non-persistent insecticides, and current research suggests that this does not include the current prescription of neonicotinoids, due to their persistence and broad-spectrum effects (Giorio *et al*., 2017). It seems that farmers and seed suppliers strongly favour an “insurance-based” approach to pest control, which suggests that IPM will not be widely adopted (Douglas and Tooker 2015). In fact, neonicotinoid seed treatments are being used outside of an IPM framework in most cropping systems (Douglas *et al.*, 2015).

Neonicotinoids have already been found to depress populations of soil-dwelling arthropod predators. The exact knock-on significance of these effects is uncertain, but a recent study by Douglas *et al*. (2015) revealed a previously unconsidered pathway for the dietary transfer of neonicotinoids from a pest slug *Deroceras reticulatum* to its predator, the beetle *Chlaenius tricolor*, with subsequent negative effects on the beetle. In the field, this depressed predator activity caused a 19% reduction in soy-bean density and a 5% reduction in yield due to a relaxation of slug predation. Similarly, thiamethoxam treated cotton seeds have also been

shown to reduce the population of natural enemies of the cotton leafhopper by about 35% (Saeed *et al*., 2017). Thus, the indiscriminate use of neonicotinoids is likely to have other unintended consequences, with measurable costs for farmers as well as biodiversity.

In January 2018, the UK Government published its "25 Year Environment Plan" (DEFRA, 2018). Referring to agriculture, they aim to put IPM at the centre of a holistic approach to encourage and support sustainable crop protection. This recognition of the importance of beneficial arthropods in agriculture could lead to the responsible use of pesticides; to focus back on IPM combined with modern machinery. The review of the UK National Action Plan for the Sustainable Use of Pesticides is also due in 2018 (DEFRA, 2018). It is vital that this opportunity to shape new agricultural policies is not wasted in a post-Common Agricultural Policy (CAP) situation and public support for the stance needs to be gained. Further, seed companies could help by increasing the availability of non-neonicotinoid treated seeds (Douglas and Tooker 2015).

### 7.6 UK Government, Brexit and Changes to the Common Agricultural Policy

The introduction of the CAP in 1962 aimed to give both a fair wage for the farmer and provide affordable food for the EU citizen. Since its inception in 1962 the CAP has undergone many changes with a continual aim to produce a more streamlined user experience for the farmer. Although greening measures have been progressively incorporated into the EU's CAP to improve environmental sustainability (Baldock *et al*. 1996), the impact of these on biodiversity recovery has been limited, with biodiversity continuing to decline (Firbank *et al*., 2008).

As a result of the referendum on the UK's membership of the European Union, on 29th March 2017, the UK Government triggered Article 50 which initiated the official process of exiting the EU, otherwise known as 'Brexit'. Currently there is uncertainty as to how Brexit will affect the farming industry, which is at present heavily supported by EU schemes. These schemes include agri-environment schemes designed to improve environmental sustainability (Maes *et al*., 2013). However, the UK's departure from the EU potentially brings with it opportunities to correct a flawed system. The Environment Secretary has plans for a "Green Brexit", where farming subsidies will only be on offer for delivering benefits to nature and the countryside, and not simply payments related to land area. Over the last few decades, agriculture has undergone an intensification of production methods which has resulted in a movement towards large-scale monoculture and farm specialisation. When farms merge, parcels of land are often blocked together into larger units to ease management; therefore the uniformity of

management has increased across large areas of land, the exact opposite of what is deemed to be beneficial for wildlife diversity (Donald *et al*, 2001)**.**

The CAP has been criticised as insulating farmers from market forces, leading to the application of 'perverse' subsidies and poor landscape-scale management. A greater focus on supporting the delivery of ecosystem services and maintaining robust, cohesive environments would be a worthy successor to the CAP, extending the UK's environmental resilience to better reflect current ecological understanding.

### 7.7 Further work

The research presented in this thesis contributed single-species toxicity assessments using chronic, field-realistic exposure levels. Despite significant interest in the field of neonicotinoid research, many gaps in knowledge remain, and my projects present areas that can be taken forwards further to improve this understanding. For all the chapters (bar Chapter 6), the experiments could be repeated with other focal neonicotinoids; work by Moffat *et al.* (2016) showed that individual neonicotinoids have been reported to have distinct binding to the nicotinic acetylcholine receptor (nAChRs) and therefore may pose differential risks to non-target organisms.

Following Chapters 2 and 5, the impact of both clothianidin and thiamethoxam on the adult stages could be investigated. This would be a simple repeat of the methodology as the experimental set up would remain the same. Further, it would be very interesting to introduce additional stressors such as sub-optimal environmental conditions, dietary limitations and pathogens to the experimental set up. While it is not practical to achieve the number of possible combinations of stressor and chemical as likely present in full field conditions (as previously discussed), investigating specific interactions to identify areas which may have particular importance to real-world outcomes has great value. In particular, the impact of neonicotinoid contaminated prey items and their entry into the wildlife food chain is an important area for future work. It is known that the consumption of neonicotinoid contaminated prey reduces the efficacy of predators' immune systems (Pisa *et al.* 2015) and so it is important to evaluate the potential exposure risks to those prey.

## 7.8 Looking forward

The current widespread loss of insect diversity is alarming and there is an urgent need to uncover the causal factors in this decline, its geographical extent, and the knock-on consequences for ecosystems. Insects play essential roles in pollination, herbivory and detrivory, nutrient cycling and providing a food source for higher trophic levels such as birds and mammals, so reduction in their populations will have cascading effects on food-webs and multiple ecosystem services (Hallmann *et al*., 2017).

Species loss can also lead to ecosystem simplification, where loss of ecological interactions are the cause of further secondary extinctions. Work by Sanders *et al* (2018) showed that the probability of secondary extinctions was higher in smaller food webs; in their example, harvesting one species of parasitoid wasp led to the extinction of other, indirectly linked species, at the same trophic level. These secondary extinctions can lead to further simplification, making the system less robust and less able to continue functioning through ecological changes (particularly climate change, or introduction of non-native species), and even leading to run-away extinction cascades. Therefore, the conservation of a wide range of species is needed to maintain stable ecosystems, as different species react differently to changes and new species may become important ecological keystones in the future (Pisa *et al*., 2017).

Neonicotinoid pesticides are heavily implicated in contributing to the global loss of insect biomass. On the 28th February 2018, the European Food Safety Authority (EFSA) published a statement concluding that most uses of neonicotinoids (imidacloprid, thiamethoxam, and clothianidin) represent a risk to wild bees (EFSA 2018). These update the conclusions published in 2013 and include consideration of solitary bees and bumblebees, as well as the previously studied honeybee. This strengthens the case for even further restrictions on the use of neonicotinoids. However, the decision regarding the regulation of pesticides lies with the European Commission and Member State authorities, not with the scientific risk assessment body EFSA. There is the need to consider that the ban will have to be extended and broadened to cover all risk pathways to protect non-target organisms other than bees and that robust scientific evidence will be critical to influence the decisions.[2] For those countries outside the

[2] As previously stated, in late April 2018, EU member states voted in favour of an almost complete ban on the use of neonicotinoid pesticides across the EU. Currently, neonicotinoids will only be able to be used in greenhouse conditions across the EU. However, the use of neonicotinoids continues unabated in many other countries despite the ban in the EU, including mass-use in the US. It is hoped that the research undertaken in Europe will inform decision making in those countries that continue to use neonicotinoids.

EU, there is a need to take advantage of the scientific advice and consider a re-think to their current pesticide regimes.

Ecosystems that are already impacted by neonicotinoid contamination need to be allowed to recover, and still deliver the functions and services they produce in the meantime. Investigations into this area of research should be a priority. Much of the major research focus on neonicotinoids has been on their effect on bees – which are crucial pollinators – but effort also needs to remain focused on the "less charismatic" yet equally essential invertebrates. The investigations reported in this thesis highlight that other important invertebrates are indeed at risk from neonicotinoid exposure, and that the use of relatively simple laboratory models can help to identify which species are most at risk and hence require further investigation. Success in halting biodiversity declines is contingent on the co-operation between organisations from several sectors, combined with education of politicians, the farming community, and the public on the concept of how their choices can impact a healthy ecosystem.

The global decline of insects as a result of human activities shows no sign of plateauing (Hallmann *et al*., 2017) and there is no time to procrastinate on this issue. There is simply too much at stake.

# **Chapter 8** – References

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